UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE AND MATHEMATICS

School of Chemistry

Synthesis of the small peptide analogues of Cyclin Dependent Kinase (CDK4) for cancer treatment

by

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ABSTRACT

FACULTY OF ENGINEERING, SCIENCE & MATHEMATICS

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Doctor of Philosophy

SYNTHESIS OF THE SMALL PEPTIDE ANALOGUES OF CYCLIN DEPENDENT KINASE (CDK4) FOR CANCER TREATMENT

by Jariya Romsaiyud

Cyclin-dependent kinases (CDKs) are a group of enzymes that are involved in cell cycle progression regulation. The CDKs activate host proteins through phosphorylation on serine or threonine using adenosine triphosphate as a phosphate donor. Especially, cyclin-dependent kinase 4 (CDK4) has attracted much attention as a potential therapeutic target in treating cancer because it is the key player in the control of cell proliferation. Comparison of the best model of CDK4 with the structures of CDK6 and CDK2 is shown difference in the cyclin-binding region and in overall electrostatic potential. A partially hydrophobic, externalized loop structure present in CDK4, but absent in CDK2 and CDK6 is identified. The hypothesis is that should CDK4 be involved in binding to an additional unidentified protein partner, this fragment provides the most likely candidate for the binding site. This has lead to the discovery of a peptide **1**, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, from a previously uncharacterized structural domain on CDK4. In this work, solution phase peptide synthetic method is optimized and developed to synthesize linear peptide **64**, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe. A series of side chain modification peptides of compound **64** was synthesized and found that peptide **71**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe had the most potent for anticancer activity. Therefore, alanine scanning compounds of hexapeptide **71** were synthesized by replacing each amino acid residue with *L*-alanine to investigate which amino acid residue had shown anticancer activity. Solid phase method was also optimized to synthesized peptide **1** and its alanine scanning compounds. To improve anticancer activity, cyclic peptides are synthesized by solution phase method. Biological assays were optimized. Clonogenic assay was chosen to test with our synthetic peptides against RT112 bladder cancer and MRC5-hTERT fibroblast cells.

To Mom and Dad

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PREFACE

The research contained within this thesis was carried out under the supervision of Professor J. D. Kilburn and Dr. Jeremy Blaydes and Dr. Sally Dixon at the University of Southampton between October 2006 and October 2009. No part of this thesis has previously been submitted to this or any other University.

DECLARATION OF AUTHORSHIP

I, Miss Jariya Romsaiyud, declare that the thesis entitled 'synthesis of the small peptide analogues of cyclin dependent kinase (CDK4) for cancer treatment' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;

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- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

- I have acknowledged all main sources of help;

- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

- none of this work has been published before submission, or [delete as appropriate] parts of this work have been published as: [please list references]

Signed: ……..

Date:……..

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Jariya Romsaiyud

ABBREVIATIONS

For chemistry part:

Ac_2O	Acetic anhydride
AcOH	Acetic acid
AIBN	α, α'-Azoisobutyronitrile
AOP	7-azabenzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluoro phosphate
BOC	*tert*-Butyloxycarbonyl
BOP	Benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluoro phosphate
BOPCl	Bis(2-oxo-3-oxazolidinyl)phosphonic chloride or Phosphoric acid bis(2-oxooxazolidide) chloride
BTBO	1,1'-Bis[6-(trifluoromethyl)benzotriazolyl]oxalate
n-Bu_4NF	*tetra-n*-Butylammonium fluoride
Bu_3SnH	Tributylstannane
CCl_4	Carbontetrachloride
CH_3CN	Acetonitrile
CH_2N_2	Diazomethane
Chloranil	2,3,5,6-tetrachloro-*p*-benzoquinone
$(COCl)_2$	Oxalylchloride
Cr_2O_3	Cromium(III) oxide
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane (CH_2Cl_2)
DCU	dicyclourea
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one
DIC	Diisopropylcarbodiimide
DIPEA	*N,N'*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide

DPPA	Diphenoxyphosphoryl azide
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EtOH	Ethanol (C_2H_5OH)
Et_2O	Diethyl ether
Fmoc	9-Fluorenyl methyloxycarbonyl
HAPyU	1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4, 5-*b*]pyridin-1-ylmethylene) pyrrolidinium hexafluorophosphate *N*-oxide
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate
HCl	Hydrochloric acid
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxy benzotriazole
HOSu	*N*-Hydroxysuccinimide
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectroscopy
H_2SO_4	Sulfuric acid
IR	Infrared
KCN	Potassium cyanide
K_2CO_3	Potassium carbonate
KI	Potassium iodide
KOH	Potassium hydroxide
LRMS	Low resolution mass spectroscopy
MeOH	Methanol (CH_3OH)
$MgSO_4$	Magnesium sulfate
MsCl	Methanesulfonyl chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NEt_3	Triethylamine
NH_3	Ammonia
NH_4OH	Ammonium hydroxide
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
PCl_3	Phosphorus trichloride

PCl_5	Phosphorus pentachloride
Pd/C	Palladium on carbon
PEG	Polyethylene glycol
Pfp-ester	Pentafluorophenyl ester
Pfp-OH	Pentafluorophenol
PhMe	Toluene
PNP	*p*-Nitrophenyl
$POCl_3$	Phosphorus oxychloride
PPh_3	Triphenylphosphine
$(PrPO_2)_3$	Propylphosphonic anhydride
PyAOP	7-Azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluoro phosphate
PyBOP	Benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate
RSPS	Repetitive (or continuous) solution phase peptide synthesis
$SOCl_2$	Thionylchloride
SPPS	Solid phase peptide synthesis
TAPipU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-pentamethylenuronium tetrafluoroborate
TBDMSCl	*tert*-Butyldimethylchlorosilane
TBTU	*O*-benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate
TDBTU	2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
THF	Tetrahydrofuran
TIS	Triisopropylsilane
TFA	Trifluoroacetic acid
p-TsOH	*p*-Toluenesulfonic acid
Ugi reaction	Multicomponent reactions
UV	Ultraviolet

For biological part:

anti-CDK1	antibody to Cyclin-dependent protein kinase 1 (first antibody)
DMEM	Dulbecco's Modified Eagle Medium
ECAC	European Collection of Cell Cultures, a Health Protection Agency Culture Collection.

EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal Calf serum
Ham's F-12 medium	Nutrient Mixture F-12 Medium
HBSS	Hank's Balanced Salt Solution
PBS	Phosphate Buffered Saline
SAR	Structure activity relationship
TLC	Thin layer chromatography
Triton X-100	Triton nonionic surfactant X-100

TABLE OF PEPTIDES

Peptides	Number
N-Boc-*L*-Arg(NO_2)-Gly-OMe	**56**
H-*L*-Arg(NO_2)-Gly-OMe	**57**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-OMe	**58**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa	**59**
N-Boc-*L*-Arg(NO_2)-*L*-Pro-OMe	**60**
H-*L*-Arg(NO_2)-*L*-Pro-OMe	**61**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**62**
H-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**63**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**64**
N-Ac-*L*-Arg(NO_2)-Gly-OMe	**65**
N-Ac-*L*-Arg(NO_2)-Gly-C(O)-NH_2	**66**
N-Ac-*L*-Arg(H)-Gly-C(O)-NH_2	**67**
N-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe	**68**
H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**69**
H-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe	**70**
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**71**
N-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-OMe	**72**
N-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-ONa	**73**
N-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**74**
H-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**75**
N-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**76**
N-Boc-*L*-Ala-Gly-OMe	**77**
H-*L*-Ala-Gly-OMe	**78**
N-Boc-*L*-Pro-*L*-Ala-Gly-OMe	**79**
N-Boc-*L*-Pro-*L*-Ala-Gly-ONa	**80**
N-Boc-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**81**
H-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**82**
N-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**83**

N-Boc-*L*-Arg(NO_2)-*L*-Ala-OMe **84**

H-*L*-Arg(NO_2)-*L*-Ala-OMe **85**

TABLE OF PEPTIDES

Peptides	**Number**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe	**86**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-ONa	**87**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**88**
H-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**89**
N-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe	**90**
N-Boc-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe	**91**
H-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe	**92**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe	**93**
H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe	**94**
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe	**95**
N-Boc-*L*-Ala-*L*-Pro-OMe	**96**
H-*L*-Ala-*L*-Pro-OMe	**97**
N-Boc-*L*-Pro-*L*-Ala-*L*-Pro-OMe	**98**
H-*L*-Pro-*L*-Ala-*L*-Pro-OMe	**99**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe	**100**
H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe	**101**
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe	**102**
H-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe	**103**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe	**104**
H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe	**105**
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe	**106**
N-Ac-*L*-Pro-*L*-Arg-*L*-Pro-NH_2	**119**
N-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2	**120**
N-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2	**121**
N-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2	**122**
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2	**123**
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2	**124**
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2	**125**

N-Boc-*L*-Arg(NO_2)-*L*-Arg(NO_2)-OMe **142**

H-*L*-Arg(NO_2)-*L*-Arg(NO_2)-OMe x HCl **143**

TABLE OF PEPTIDES

Peptides **Number**

N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-OMe **144**

N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-ONa **145**

N-Boc-Gly-*L*-Pro-OMe **146**

H-Gly-*L*-Pro-OMe x HCl **147**

N-Boc-*L*-Pro-Gly-*L*-Pro-OMe **148**

H-*L*-Pro-Gly-*L*-Pro-OMe x HCl **149**

N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe **150**

Methanesulfonic acid 2-[2-[2-(2-hydroxyethoxyethoxy]ethoxy]ethyl ester **151**

PEG-linked azido alcohols 12 (2-[2-[2-(2-Azidoethoxy) ethoxy]ethoxy] ethanol) **152**

PEG-linked azido alcohol **153**

PEG-linked azides **154**

N-Boc protection of PEG-linked tether **155**

N-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro OMe **156**

N-PEG-*N'*- Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-ONa **157**

N-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-Opfp **158**

N-PEG-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-Opfp x HCl **159**

N,C-PEG-tethered-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro **160**

N,C-PEG-tethered-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro x AcOH salt **161**

H-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe x HCl **162**

N-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe **163**

N-PEG-*N'*- Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-ONa **164**

N-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-Opfp **165**

N-PEG-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-Opfp x HCl **166**

N,C-PEG-tethered-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro **167**

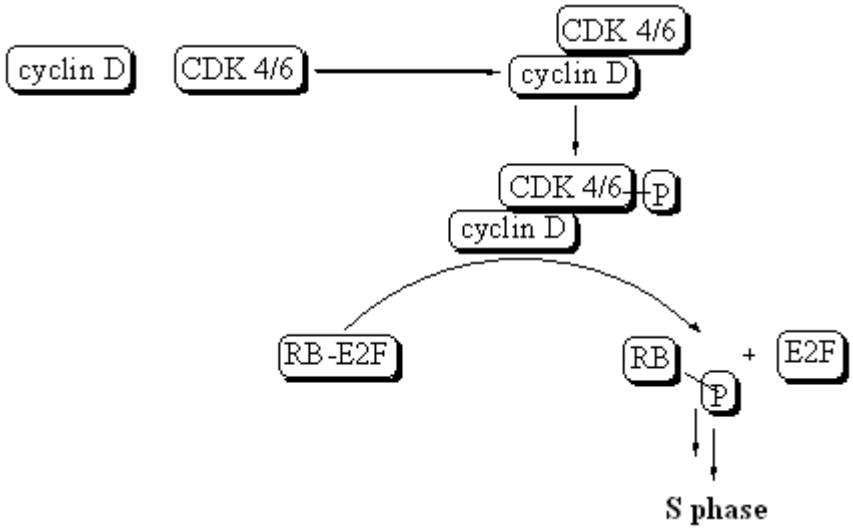
N,C-PEG-tethered-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro x AcOH salt **168**

CHAPTER 1: INTRODUCTION

1.1) BACKGROUND

Cancer has been recognized as a disease of uncontrolled cell proliferation.

Cyclin-dependent kinases (CDKs) are family of serine/threonine protein kinases. The CDKs play important roles in the regulation of cell division and form complexes with their activating partners such as the cyclins, in order to be active [1]. CDK/cyclin complexes can act as switches that regulate each of the cell cycle transitions through four distinct phases: G1, S, G2, and M [2]. In view of the strong relationship between CDKs and the cause of cancer, CDKs are investigated as possible targets for cancer chemotherapy [3]. Especially, CDK4 is an important enzyme which has been implicated in the development of cancer cells. Along with CDK2 and CDK6, these enzymes are traditionally thought to be responsible for the hyper-phosphorylation of retinoblastoma protein (Rb), that is the signal to start the cell division and the transition of the cell from the S to the G1 phase (scheme 1) [4].

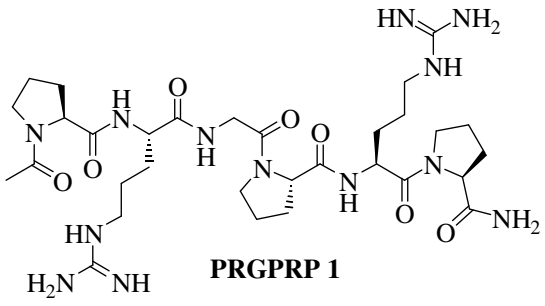


Scheme 1: The phosphorylation pathway in CDK 4.

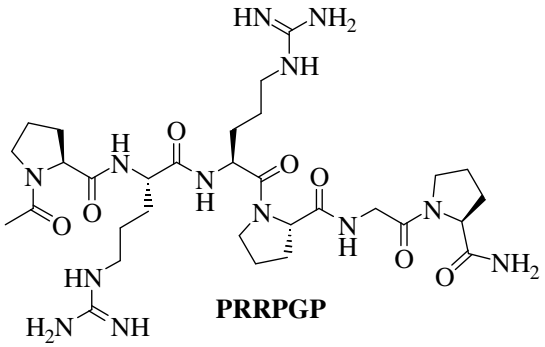
Warenius *et al.* [5] described a unique relationship between proteomic expression of CDK1 and CDK4 in human cancer cells. The over expression of CDK4 in cancer cell lines resulted an increase of CDK1 protein levels, in the absence of any evidence for an increase in CDK4 dependent phosphorylation of Rb protein [6], [7]. The model study of CDK4 has

been studied by Dr. J. Essex [8]. The investigation of novel function domains of CDK4 is compared with structure of CDK6 and CDK2 and finds that CDK4 contains both α/β structure similar to the structure of CDK6 and CDK2. A partially hydrophobic, unique, externalized loop structure that present in CDK4, but absent in CDK2 and CDK6 is identified as amino acid sequence FPPRGPRPVQSV [9]. Biological study of this peptide was done by clonogenic assay. Human bladder cancer cells (RT112) was exposed to a 1 mM solution of the peptide and found that 30-50% cells are dead over the first few days. Subsequently, the cells recovered and grew relatively normally until 21-25 days after the start of the experiment. Furthermore, shorter peptide sequences of linear peptides such as PRGPRP, PRGPR, RGPRP, RGPR, TRGPRP, TRGTRP, PRGPRT and PRGTRT are exposed to RT112 bladder cancer and normal fibroblast cell lines (5 mM concentration of peptide solutions). One key finding from this study reveals that the hexapeptide sequence PRGPRP is the most selective and effective moiety being non-toxic to normal fibroblasts but selectively killing RT112 bladder cancer cells at a concentration of 5 mM. On the other hand, hexapeptide sequence PRRPGP does not show any biological activity with cancer cell lines. These suggest that, although 5 mM of peptide solution that is used to detect the PRGPRT selective anticancer activity is very high concentration, the effect of this particular hexapeptide sequence is specific. The selective effect of cancer cell killing by PRGPRP as opposed to PRRPGP is confirmed by clonogenic assay that has already developed and reported by Warenius, H. *et al*. [10]. The chemical structures of both PRGPRP and PRRPGP are shown in scheme 2.

Note: P = Proline, R = Arginine, G = Glycine, T = Threonine.



PRGPRP 1

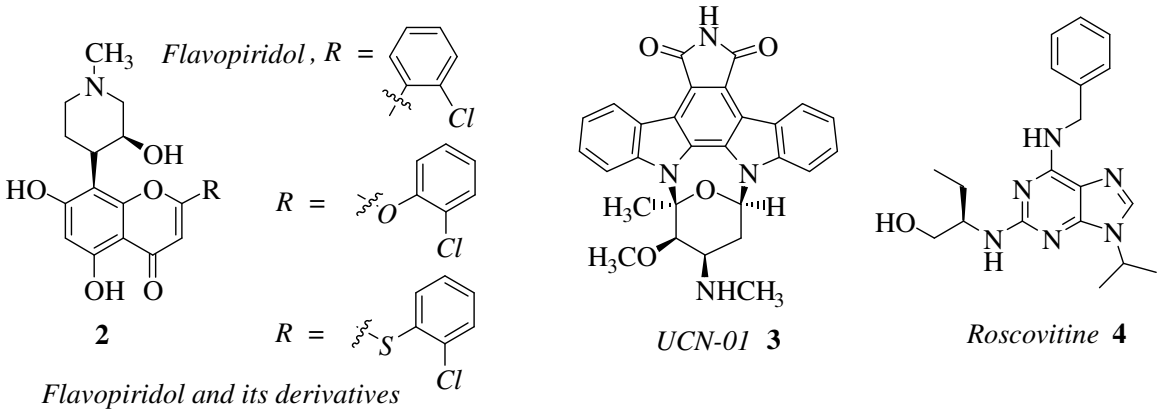


PRRPGP

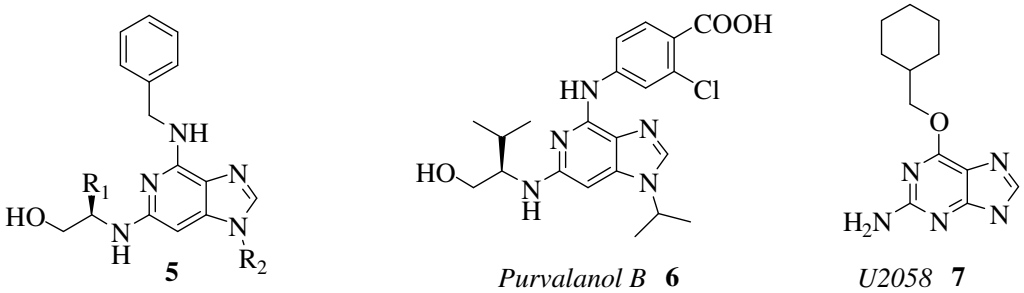
Scheme 2: Chemical structures of PRGPRP (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg-*L*-Pro-NH_2) **1** and PRRPGP (*N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro-NH_2).

1.1.1) Literature reviews of CDK inhibitors.

During the past decade, several small molecules were discovered for the inhibitory activity against CDK such as flavopiridol [11], UCN-01 [12] and roscovitine [13] (scheme 3). These three compounds had been entered to clinical development as cancer theraputics with broad spectrum of CDK inhibitors. Especially, the derivatives of flavopiridol that were synthesized to contain *oxo-* or *thio-* group in the molecule, had been improved selectivity over CDK2 and CDK4 [14]. In 2000, Yu *et al.* [15] investigated substituted guanines and pyrimidines families such as olomoucine, roscovitine, purvalanol B and U2058 (scheme 4) which obtained better specificity of CDK inhibitor. In 2003, Watkins *et al.* [16] successfully synthesized unsymmetrically substituted indolocarbazole compounds (SB-210878 and arcyriaflavin A) for CDK4/D1 inhibitor. The key step in the synthesis is involved the cyclization of an intermediate **A** to the product **B** by DDQ-mediated oxidative cyclization [17] in the presence of a catalytic amount of *p*-TsOH or oxidation by iodine under photochemical conditions (scheme 5).

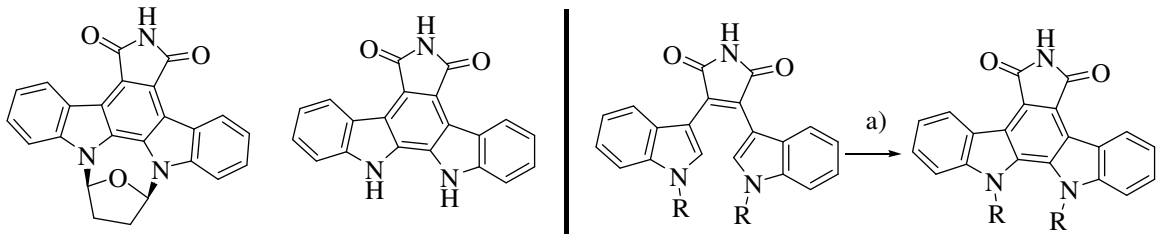


Scheme 3: Chemical structures of CDK inhibitors.



Olomoucine: $R_1 = H$, $R_2 = CH_3$
Roscovitine, $R_1 = CH_2CH_3$, $R_2 = CH(CH_3)_2$

Scheme 4: Chemical structures of CDK inhibitors.

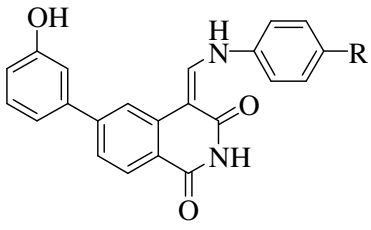


SB-210878 **8** Arcyriaflavin B **9** **A** **B**

a) Key cyclization step: DDQ, solvent, p-TsOH (catalytic amount), reflux or I_2, benzene, hv.

Scheme 5: Chemical structures of CDKs inhibitors and the key cyclization step of indolocarbazoles synthesis**.**

In 2008, Rabindran *et al.* [18] successfully synthesized 4-(phenylaminomethylene) isoquinoline-1,3-(2*H*,4*H*)-dione and its derivatives as a novel class of inhibitors that selectively inhibited CDK4 over CDK2 and CDK1. Binding study found that there was hydrogen bonding interaction between 4-(phenylaminomethylene) isoquinoline-1,3(2*H*,4*H*)-dione derivatives and amino acid residues of binding pocket of CDK4. The model structure of CDK4 that was used in this work was developed by Ikuta *et al.* [19]. The chemical structure of 4-(phenylaminomethylene) isoquinoline-1,3(2*H*,4*H*)-dione and its derivatives are shown in scheme 6.



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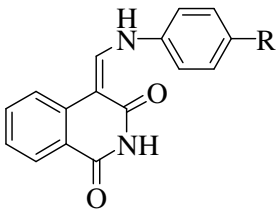
R= 4-Me-piperazinyl or CH_2-(1-piperidinyl)



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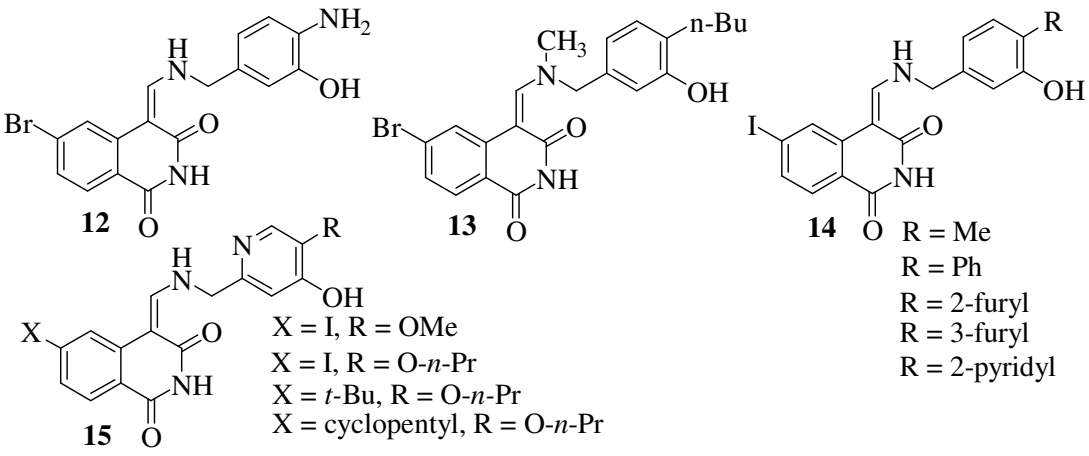
R= 4-Me-piperazinyl or 4-morpholinyl or CH_2-(4-morpholinyl) or CH_2-(1-piperidinyl)

Scheme 6: Chemical structures of 4-(phenylaminomethylene) isoquinoline-1,3(2*H*,4*H*)-dione and its derivatives.

Recently, a novel series of 4-(phenylaminomethylene)isoquinoline-1,3-diones is reported as a potent and selective inhibitors of CDK4. An *N*-methylpiperazine or a piperidinyl methyl group substitution at the *para* position of the aniline is essential for CDK4 potency [20] (scheme 7).



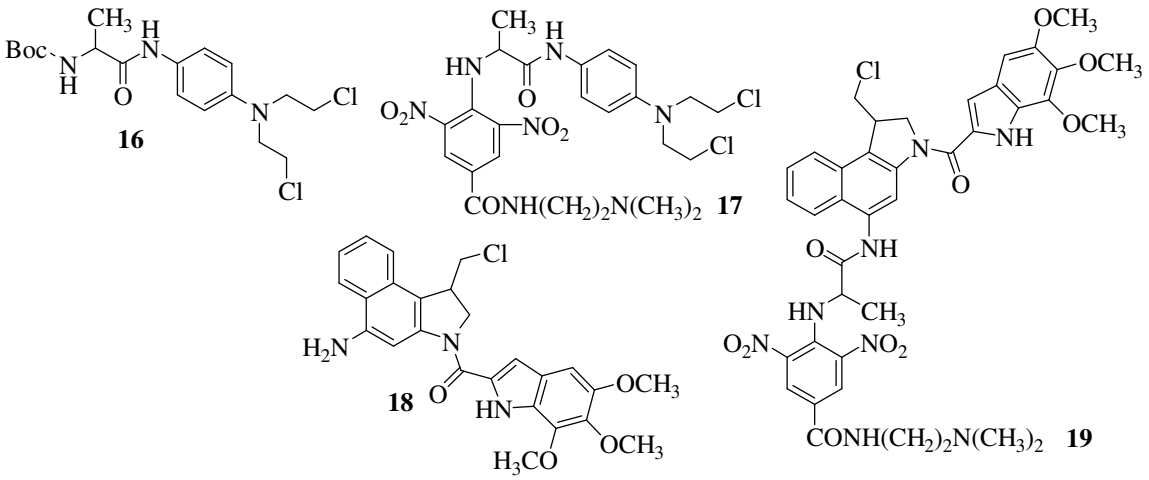
Scheme 7: The chemical structures of 4-(benzylaminomethylene)-isoquinoline-1,3-(2*H*,4*H*)-dione and 4-[(pyridylmethyl)-amino methylene] isoquinoline-1,3-(2*H*,4*H*)-dione derivatives.

1.1.2 Literature reviews of clonogenic assay.

Clonogenic assay is a widely used approach in pharmaceutical, clinical and basic research for testing the effects of drugs on the growth and proliferative characteristics of cells *in vitro*. Cytotoxicities of synthetic compounds are determined by clonogenic assay and IC_{50} values are calculated from this assay as the drug concentration providing 50% inhibition of growth relative to controls on the same plate. This technique is used to evaluate the difference in colony forming ability of cultures grown under control and test conditions. In order to obtain an accurate quantitative treatment effects in clonogenic assays, a precise method for visualizing and counting cell colonies is required. In general, cancer cells are trypsinized and seeded in tissue culture plates and allowed to grow for 24 h before exposure to drugs. Drug stock solutions are prepared in DMSO and diluted into culture medium (final DMSO concentration < 1%). The cells are exposed to drugs and kept in an incubator. Control cells are only incubated in the culture medium. After incubation time, the cells are fixed and stained using suitable methods.

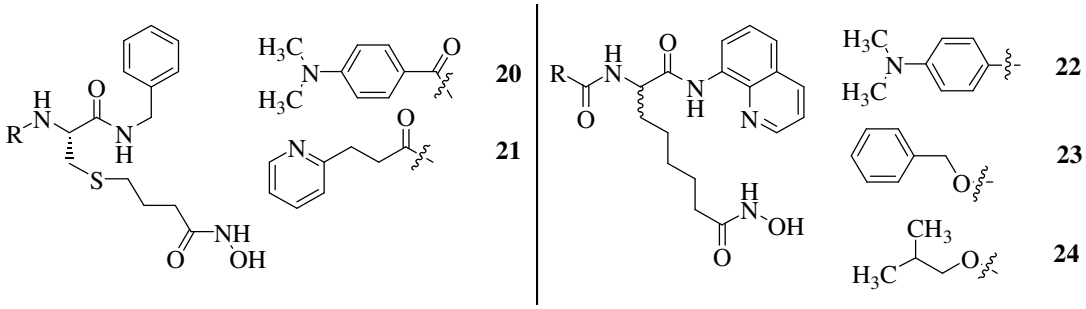
A series of amine compounds **16**-**19** was prepared and the anticancer activity of these compounds was studied and measured by clonogenic assay [21]. The chemical structures of compounds **16**-**19** are shown in scheme 8. The cells were exposed to drugs for 4 h in an incubator and then drugs were removed by washing cultures three times with fresh

medium. The cells cultures were incubated in medium for a further 4-5 days before staining with methylene blue [22].



Scheme 8: Chemical structures of amine compounds **16**-**19**.

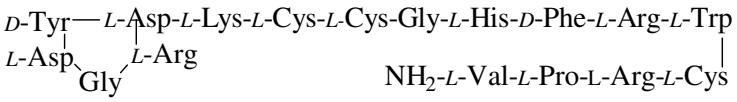
A series of hydroxamic acid was prepared and the cytotoxicities were determined by clonogenic survival of human cancer (MM96L, melanoma) and normal cells [23]. The chemical structures of hydroxamic acid and its derivatives are shown in scheme 9. The cells cultures were allowed to expose to drug solutions for 24 h and then drugs were removed by washing cultures three times with fresh medium. The cells cultures were incubated in medium for a further 4 days. The cultures were fixed with trichloroacetic acid and stained with sulforhodamine B (SRB) [24].



Scheme 9: Chemical structures of hydroxamic acid and its analogous **20-24.**

Cytotoxicity of RGD-Lys-(Arg[11])CCMSH **25** was studied and measured by clonogenic assay against B16/F1 melanoma cells [25]. The chemical structures of compounds **25** are shown in scheme 10. The cells were exposed with drug for 3 h, washed and allowed to form colonies over 6 days in the culture medium. The medium was changed every other

day. After 6 days, the cells were fixed with methanol/glacial acetic acid (3:1) and stained with hematoxylin



Scheme 10: Chemical structure of peptide**,** RGD-Lys-(Arg^{11})CCMSH, **25**.

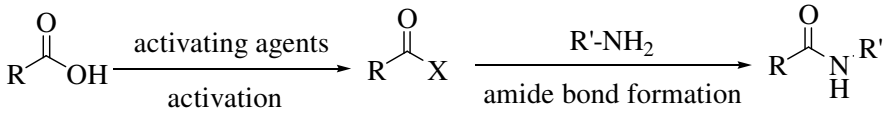
The cells cultures could be visually examined under microscope for survival (colonies contained more than 50 cells were scored as survivors) or scored by a colony counter (such as BioTran III, New Brunswick Scientific Co., Edison, NJ).

1.2) PEPTIDES SYNTHESIS

The synthetic methodology for creating peptides is among the most highly developed synthetic methodologies in chemistry [26].

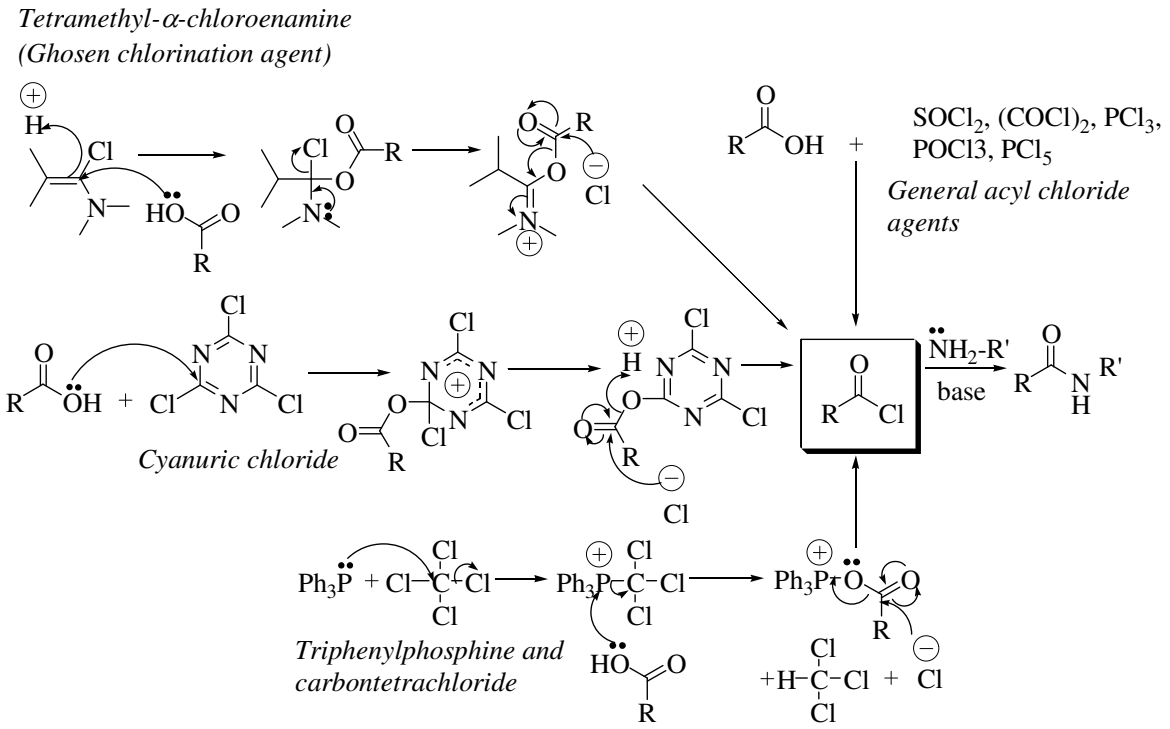
1.2.1) Literature reviews of coupling reagents and solution phase peptide synthesis.

The solution phase peptide synthesis is a classical synthesis method and has become a well established science in the field of organic chemistry. Well designed protecting groups, activating reagents and coupling reagents have been developed and improved during decades of research. It retains value in large-scale manufacturing and for specialized laboratory applications. However, the need to optimize reaction conditions, yield and purification procedures for essentially every intermediate (each of which has unpredictable solubility and crystallization characteristics) renders classical methods time consuming and labour intensive. Amide bond formation that is formally condensation between an acid and amine, can often present difficulties such as low yields, racemization and difficult purification. To solve all of these problems, numerous coupling reagents and methods have been developed to improve % yield and prevent racemization of neighboring chiral centres. A classical racemization is described in chapter 2. Especially the amide bond formation for peptides synthesis, *N*-protected-amino acid is activated under mild conditions to generate intermediate which contains good leaving group and allows to attack by amino group from another amino acid (scheme 11).



Scheme 11: General route for amide bond formation.

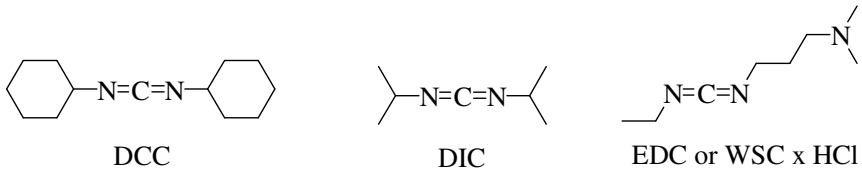
There are a lot of reagents for *C*-terminus activation which are developed and summarized: *Acyl chloride formation* is one of the easiest methods to activate *C*-terminus of *L*-amino acid. There are a lot of acid chloride agents such as thionyl chloride ($SOCl_2$) [27], oxalyl chloride $(COCl)_2$ [28], phosphorus trichloride (PCl_3) [29], phosphorus oxychloride ($POCl_3$) [30], phosphorus pentachloride (PCl_5) [31], cyanuric chloride [32], triphenylphosphine and carbontetrachloride (PPh_3/CCl_4) [33] and tetramethyl-α-chloroenamine (Ghosez chlorination agent) [34] which can be used for activation step (scheme 12).



Scheme 12: Summary of acyl chloride formation.

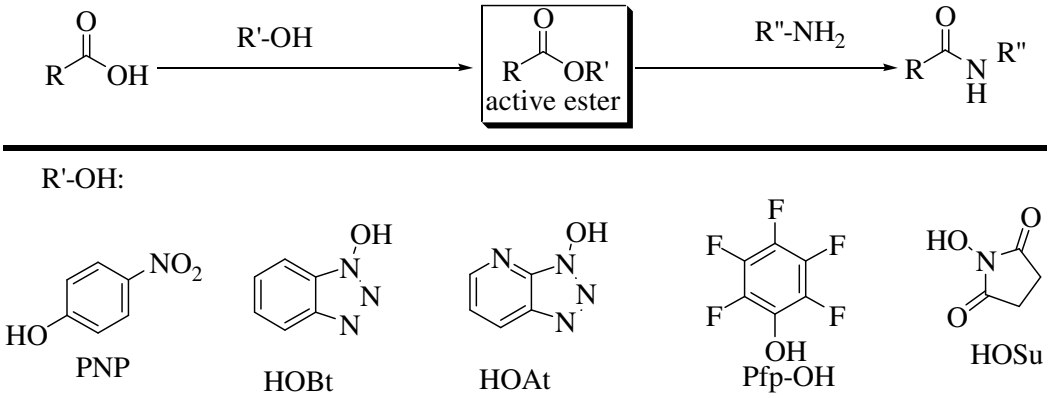
Carbodiimides such as DCC [35], DIC and EDC (or WSC x HCl) are often used as coupling reagents to generate *O*-acylisourea intermediate which can then directly react with the amino group to yield amide bond for peptide synthesis. Both of the DCC and EDC are used in solution phase peptide synthesis. Resulting urea from DCC is water-insoluble and can be removed from reaction mixture by filtration. On the other hand, the urea forming

from EDC is water soluble and can be eliminated from reaction mixture by washing. DIC is frequently used in solid phase peptide synthesis and resulting urea is water soluble which can easily be removed from reaction mixture by washing (scheme 13).

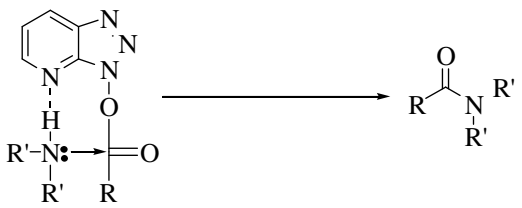


Scheme 13: Commonly used carbodiimides.

Active esters are more recently used to increase an electrophilicity of carbonyl centre. Especially in peptide synthesis, HOBt is the most commonly used to activate amino group of peptide chains or amino acids under mild conditions and also reduce racemization. The Pfp-OH [36] has been recommended for the preparation of heterocyclic peptides where DCC or DIC on its own has failed [37]. The summary of alcohols which is used to generate active ester is shown in scheme 14. HOAt has been reported to be more efficient than HOBt because its increase the chelation or add the neighbouring effect which provide by the pyridine nitrogen during the aminolysis step [38] (scheme 15).

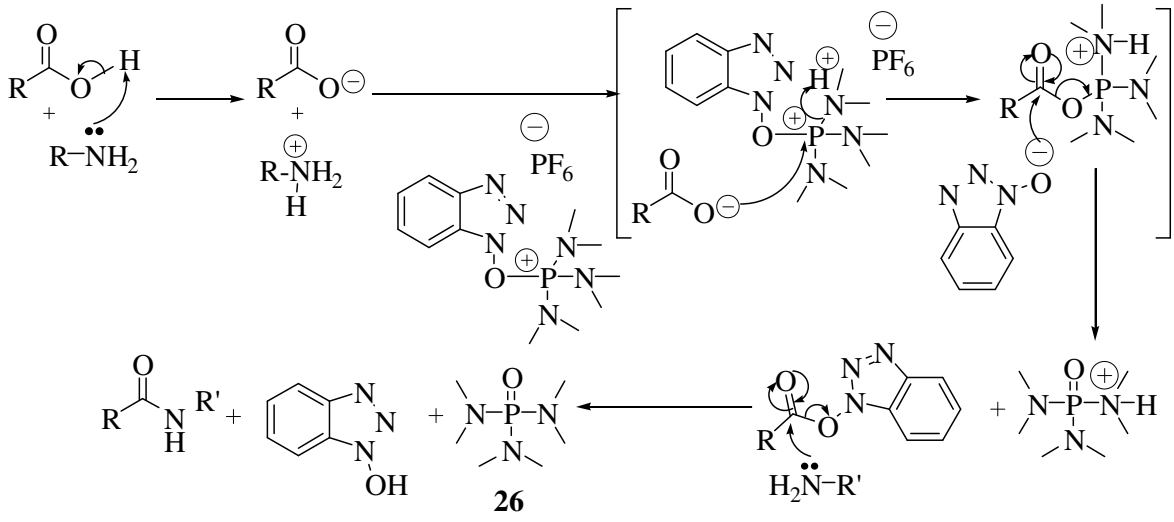


Scheme 14: Summary of active ester formation.

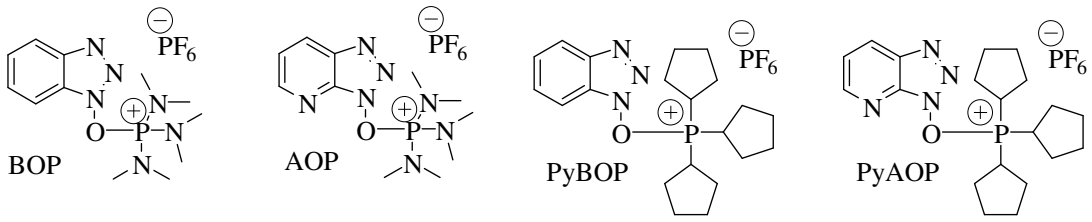


Scheme 15: The addition of chelation by HOAt.

Phosphonium-based coupling reagent: In 1975, B. Castro*, et al*. synthesized and published the first example of benzotriazol-1-yl-oxytris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) or Castro's reagent [39]. This HOBt-based onium salt reagent can be performed in one-pot coupling of the mixture between acid and amino residues under basic conditions. The carboxylate is reacted with BOP to generate both an activated acylphosphonium species and HOBt. This HOBt readily reacts with the activated acid to produce a reactive Bt ester, which finally undergoes aminolysis. The driving force of this phosphonium-based reaction is to generate the corresponding oxide **26** (scheme 16) [40]. The commonly phosphonium-based coupling reagents are summarized in scheme 17.

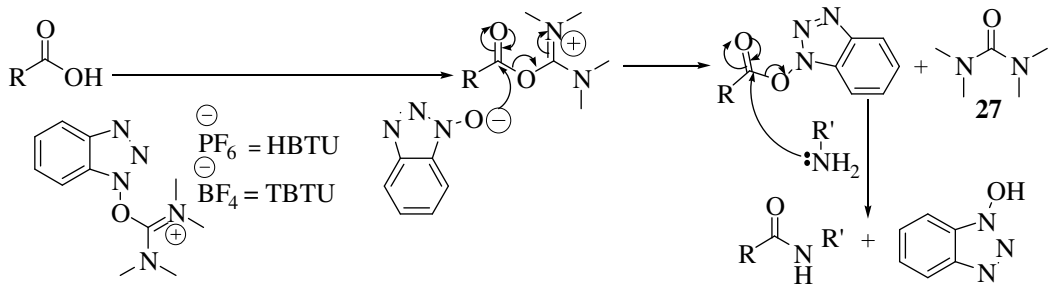


Scheme 16: One-pot coupling procedure using BOP**.**



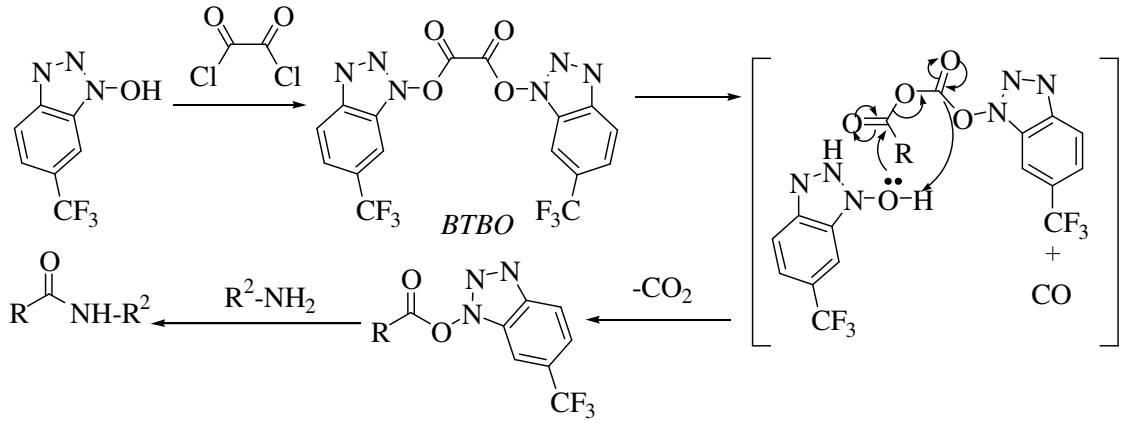
Scheme 17: Phosphonium-based coupling reagents.

Uronium/guanidium-based coupling reagents [41]: The family of uronium salt reagents has been developed around uronium species such as HBTU [42] and its tetrafluoroborate equivalent TBTU [43]. The coupling reaction is performed in a similar way to that using the phosphonium species. In this case, the driving force is the generation of the urea by-product **27** (scheme 18).



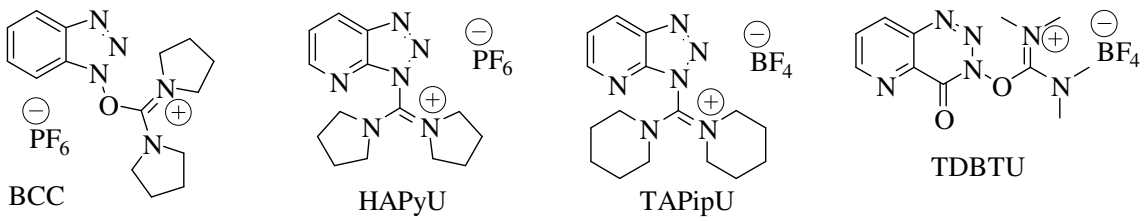
Scheme 18: Mechanism of amide bond formation by uronium/guanidium-based coupling reagents**.**

1,1'-Bis[6-(trifluoromethyl)benzotriazolyl]-oxalate (BTBO) that is easily prepared from 1-hydroxy-6-(trifluoromethy1) benzotriazole and oxalyl chloride, is used as a powerful coupling reagent for peptide synthesis [44] (scheme 19).



Scheme 19: Mechanism of amide bond formation by BTBO coupling reagent.

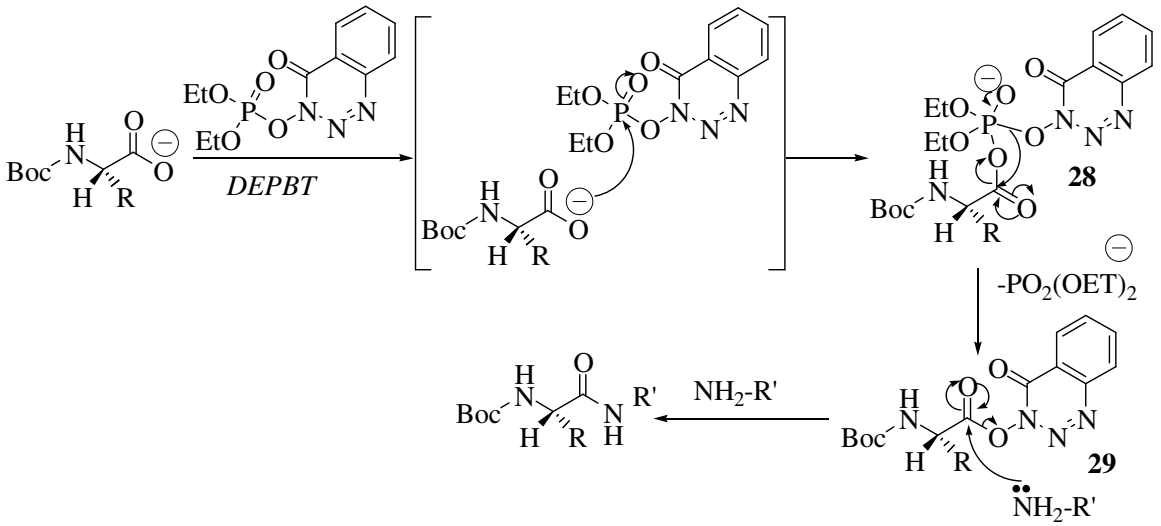
The commonly uronium/guanidium-based coupling reagents are summarized in scheme 20.



Scheme 20: Uronium/guanidium-based coupling reagents.

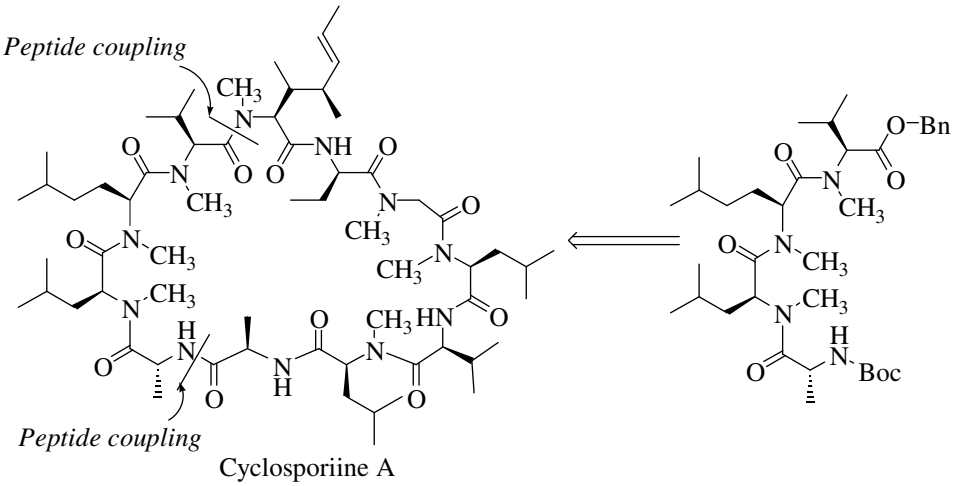
Recently, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) is known as a novel coupling reagent which is used to reduce racemization at the α-stereocenter of

activated carboxylate [45]. The purpose mechanism is shown in scheme 21. The carboxylate component is acted as a nucleophile and attached at phosphorus atom of DEPBT to obtain an unstable intermediate **28** which is easily rearranged to more stable intermediate **29** and loose of diethyl phosphite. The amino component is attached to this intermediate and formed an amide product.



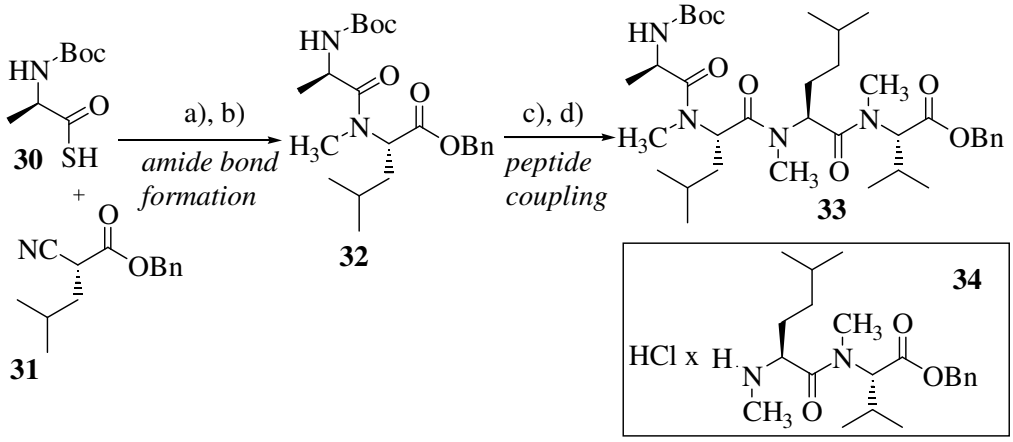
Scheme 21: Amide bond formation by activation of DEPBT.

The formation of tertiary amide bond by the use of isonitrile-mediated bond construction is currently interested in peptide synthesis especially to improving the total synthesis of biologically active polypeptides [46]. Danishefsky *et a*l. successfully generated amide bond by the use of isonitrile compound and also synthesize cyclosporine A [47-49]. The chemical structure of cyclosporine A and its tetrapeptide precursor are shown in scheme 22.



Scheme 22: Chemical structures of cyclosporine A and its tetrapeptide precursor.

The tertiary amide was smoothly formed by the coupling between thio-acid **30** and isonitrile compound **31** in $CHCl_3$ and then followed by radical reduction to obtain *N*-methylated dipeptide **32** in 59% yield over 2 steps. The tetrapeptide precursor **33** was synthesized by hydrogenolysis of the benzyl ester to provide acid compound which was subsequently coupled with amino residue **34** by DEPBT and DIPEA in THF. The desired product **9** was obtained in 90% over 2 steps with no epimerization (scheme 23).

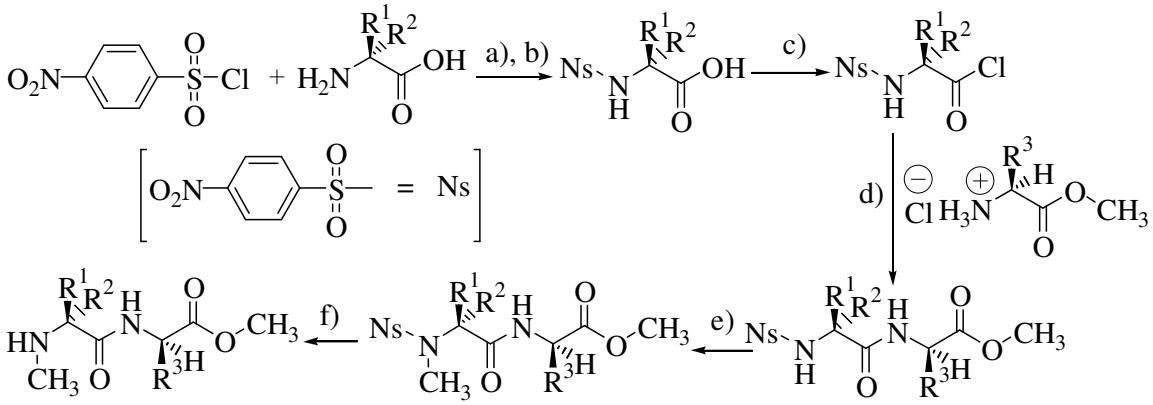


*a) $CHCl_3$, b) Bu_3SnH, AIBN, 100 °C, PhMe (59% over 2 steps), c) H_2, 10% Pd/C, MeOH, d) DEPBT, DIPEA, THF, **34** (90% over 2 steps).*

Scheme 23: Synthesis of tetrapeptide precursor **33**.

N-methyl peptide has a lot of advantages such as provide a bulky steric modification that can change conformation of peptide, block intramolecular hydrogen bonding and enhance

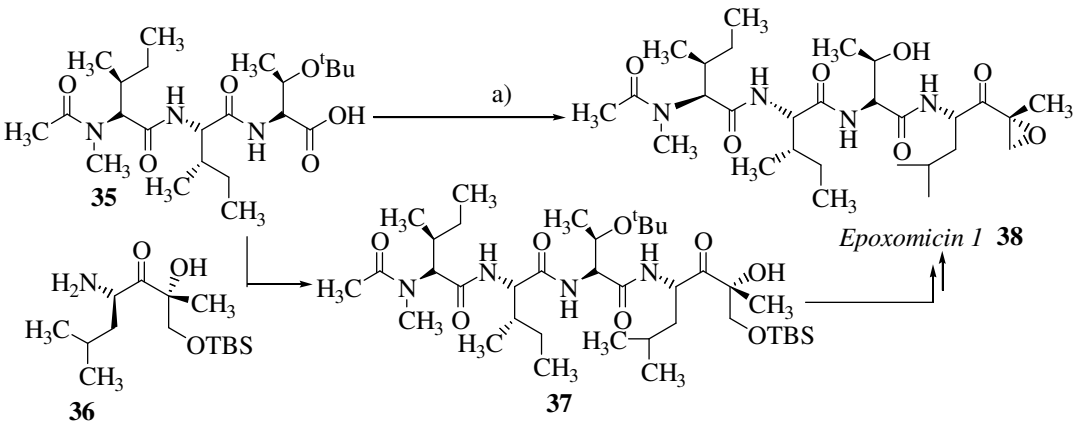
hydrophobic property of compound. Recently, naturally occurring of *N*-methyl peptides exhibit biological activities such as cyclosporines [50], dolastatins [51], didemnins [52] and epoxomicin 1 [53]. A new and efficient solution method for *N*-methylated peptide is successfully done by diazomethane at specific *N*-terminus of peptide chain [54] (scheme 24). The diazomethane is acted as base and methyl diazonium ion is generated in situ from an ethereal solution of diazomethane [55].



a) NaOH, H_2O, b) HCl, c) $SOCl_2$, CH_2Cl_2, Δ, d) $NaHCO_3$, H_2O, $CHCl_3$, e) CH_2N_2, Et_2O, f) $HSCH_2COOH$, $NaOCH_3$, 50 °C.

Scheme 24: *N*-methylation of peptide by diazomethane.

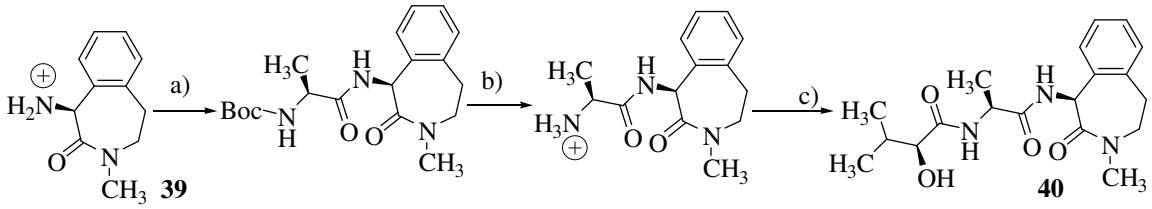
In 2004, Williams *et al.* presented an efficient route to synthesize *N*-methyl peptide **35** (epoxomicin 1) which was a proteasome inhibitor [53]. The key step of amide bond formation was done between starting materials **36** and **37** under DCC-promoted condition in the present of HOBt and DIPEA to obtain peptide precursor **38** in high % yields (scheme 25).



*a) **36**, DCC, HOBt, DIPEA, CH_2Cl_2-DMF, 86%.*

Scheme 25: The synthesis of Epoxomicin 1 **38** by peptide coupling under DCC and HOBt.

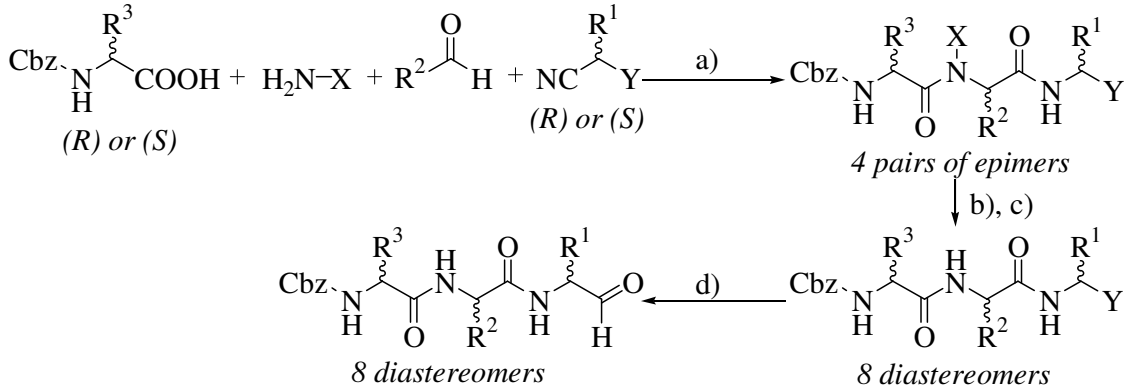
Peptide or amide bond formation is also developed in aqueous media by using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) for *C*-terminus activation and HOBt to prevent racemization. The desired product is obtained in reasonable yield [56]. Compound **39** was coupled with *N*-Boc-*s*-Ala-OH by EDC-mediated in the presence of HOBt and water. *N*-Boc protecting group was removed by concentrated HCl and followed by coupling reaction with *s*-Val-OH under the same conditions to obtain **40** (scheme 26).



a) *N*-Boc-*s*-Ala-OH, EDC, HOBt, water, b) *N*-Boc deprotection by concentrated aq. HCl, c) *s*-Val-OH, EDC, HOBt, water.

Scheme 26: Peptide bond formation in aqueous solvent.

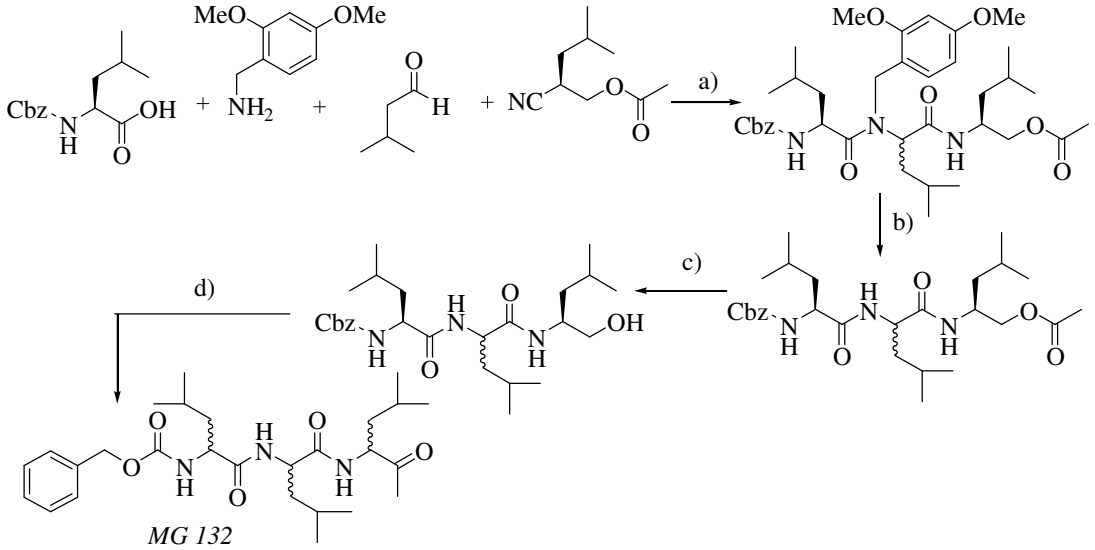
Application of Ugi reaction gives the opportunity for a quick and easy synthesis of small peptides that reveal a wide spectrum of biological activities [57]. However, this reaction is limited to the synthesis of simple peptides and according to a mechanism of Ugi reaction, the product is formed as a mixture of two epimers in difference of configuration at α-carbon. The general method for tripeptide aldehyde formation by Ugi reaction is shown in scheme 27.



a) Ugi reaction, b) separation of epimers, c) deprotection of amide group, d) carbonyl formation.

Scheme 27: General method for tripeptide aldehyde formation by Ugi reaction.

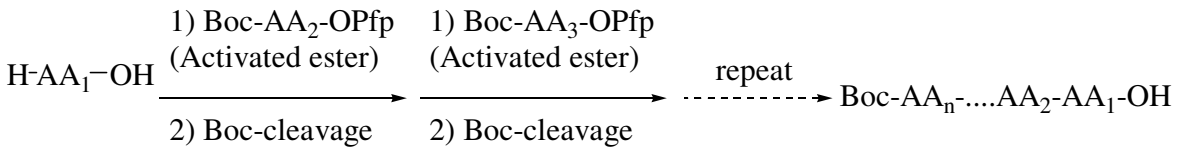
Recently, Ostaszewsk *et al.* synthesized tripeptide aldehydes such as MG132 that obtained an antitumor activity and also enhanced cytostatic/cytotoxic effect of chemo- and radiotherapy by Ugi reaction [58] (scheme 28).



a) MeOH, RT, 48 h, b) TFA/CH_2Cl_2, 50 °C, 1 h, c) aq. NaOH, MeOH, RT, 30 min, d) Dess-Martin reagent, CH_2Cl_2, RT, 1.5 h.

Scheme 28: Synthesis of MG 132 by Ugi reaction.

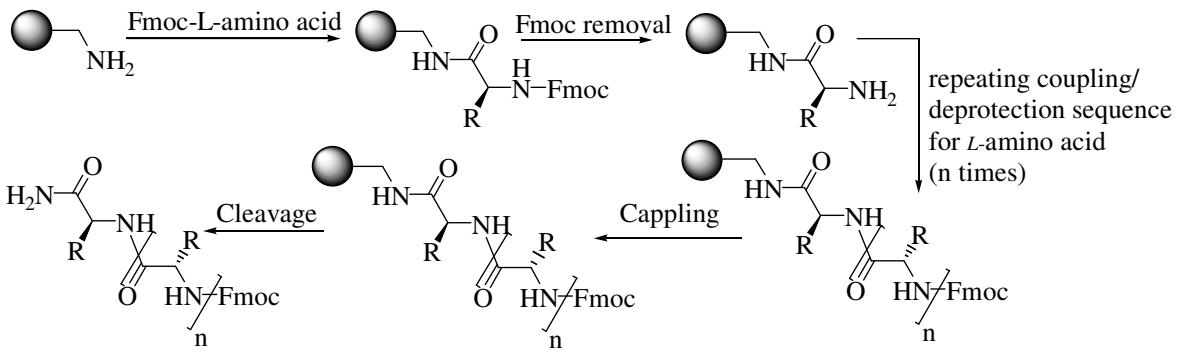
Repetitive (or continuous) solution-phase peptide synthesis (RSPS) is a novel method to prepare multigram scale peptides using *N*-Boc-protected Pfp-esters of amino acids as the activated species for coupling reactions [59]. The aims of this procedure are to synthesize peptide without any purification of intermediates and use lower quantities of expensive coupling reagent and also decrease waste which is the advantage of synthesis in large scale (scheme 29). *N*-Boc-protected pentafluorophenyl ester was prepared by coupling reaction between *N*-Boc-amino acids and Pfp-OH in the presence of EDC and then reacted with H-amino acid in the presence of DIPEA and CH_2Cl_2. Excess amino acid and by products were easily removed by washing with a 10% aq. citric acid. *N*-Boc protecting group was removed by HCl in dioxane. The coupling and *N*-Boc-cleavage cycles are repeated until the desired peptide sequence has been reached.



Scheme 29: Synthesis of peptide by Repetitive (or continuous) solution-phase peptide synthesis (RSPS).

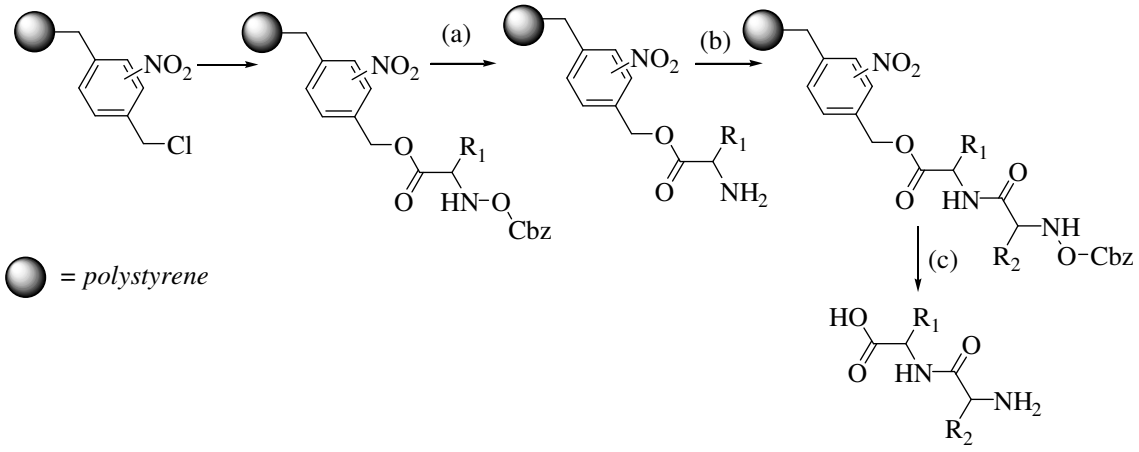
1.2.2) Reviews of solid phase peptide synthesis.

The solid phase peptide synthesis is a process by which chemical transformations can be carried out on solid support in order to prepare a wide range of synthetic compounds. During last two decades, the solid-supported chemistry has yielded numerous applications, especially in the field of parallel and combinatorial chemistry [60]. The fundamental of this technique involves the incorporation of *L*-amino acids into a peptide of any desired sequence with one end of the sequence remaining attached to a solid support matrix. While the peptide is being synthesized usually by stepwise methods, all soluble reagents can be removed from the peptide-solid support matrix by filtration and washed away at the end of each coupling step. After the desired sequence of amino acids has been obtained, the peptide can be removed from the polymeric support. The principle of polymer-supported peptide synthesis is shown in scheme 30. The first *N*-protected amino acid is attached onto resin under standard coupling conditions. *N*-protecting group is removed and coupled with second *N*-protected amino acid. This sequence is repeated until desired peptide was obtained. Finally, target peptide is cleaved from the polymer support. The solid phase chemistry offers many advantages over conventional synthesis in terms of efficiency as well as convenient work-up and purification procedures.



Scheme 30: Solid-supported solid phase peptide synthesis.

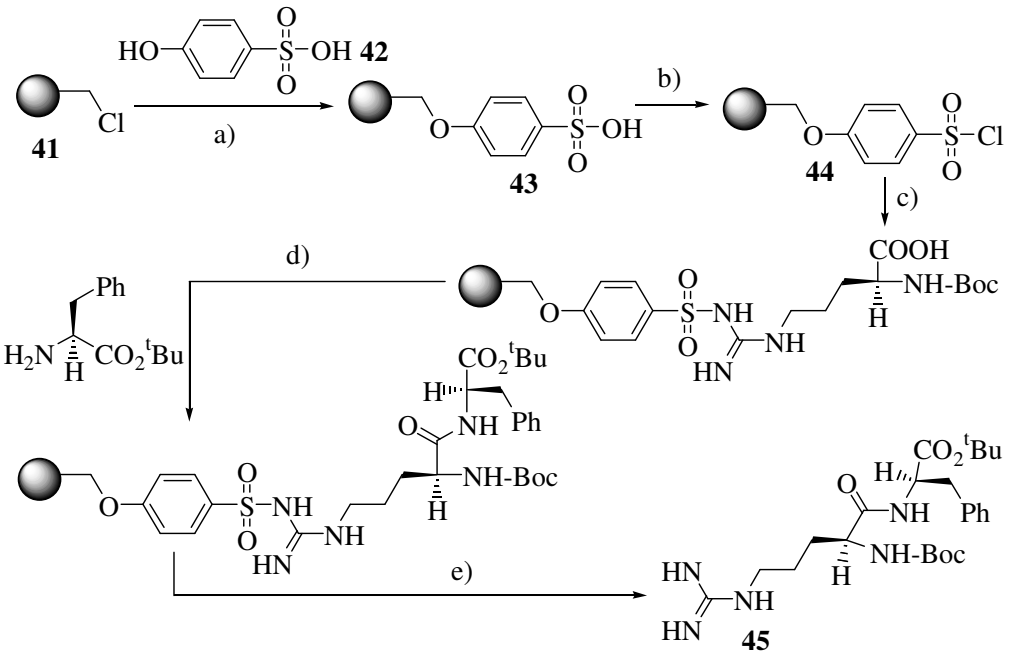
In 1963, Merrifield, R. B. successfully developed new reagents and technique to synthesize small peptides [61]. The synthetic outline is shown in scheme 31. First *N*-protected amino acid is coupled with water insoluble solid support (polystylene) by covalent bond. The great advantages of this method are simple, short time procedure and racemization of the *C*-terminal amino acid cannot be observed during the reaction. Furthermore, by products and reagents can be easily removed from the reaction mixture by washing and filtration and no need to purify by column chromatography.



a) HBr-HOAc, b) diimide, c) NaOH.

Scheme 31: General procedure of solid phase peptide synthesis.

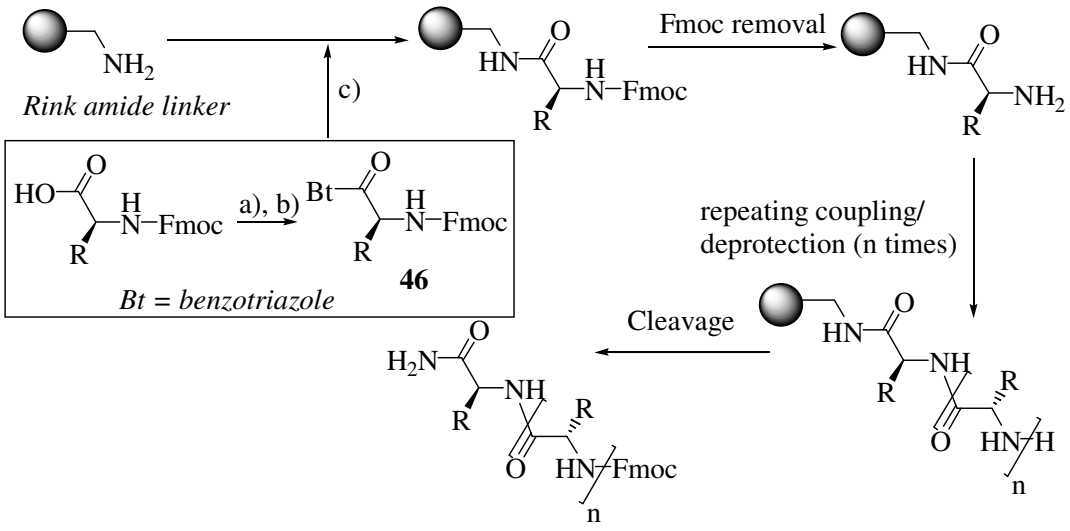
For the synthesis of arginine-containing peptides, protection of the guanidine group with *N*-nitro, *N*-arenesulfonyl, and *N*-carbalkoxy moieties are frequently required [62]. Then, in 1997, Maryanoff *et al.* investigated a series of nitrogen attachment to a resin-bound by using of arenesulfonyl as a linker that was stable, suitable and also compatible for both Boc and Fmoc peptide chemistry [63]. Merrifield resin **41** was reacted with sulfonate **42** in the presence of K_2CO_3 and KI to give benzyl ester **43** and the corresponding sulfonyl chloride **44** was prepared by using PCl_5. This novel resin-bound arginine derivative **44** was coupled with *N*-Boc-*L*-Arg(H)-OH in the presence of 4M KOH/dioxane for 2 days and followed by *N*-Boc-*L*-Phe-OH under DIC and HOBt conditions. The dipeptide **45** was cleaved from resin by HF and anisole for 4h and obtained in high yield after purification (scheme 32).



a) K_2CO_3, KI, DMF, 90 °C, 24h, b) PCl_5, DMF, 2h, c) Boc-Arg(H)-OH, 4M HCl/dioxane, 75 °C, 2 days, d) DIC, HOBt, DMF, 24 h, e) HF, anisole, 0 °C, 4h.

Scheme 32: Arginine containing peptide **45** that is synthesized by using of arenesulfonyl as a linker**.**

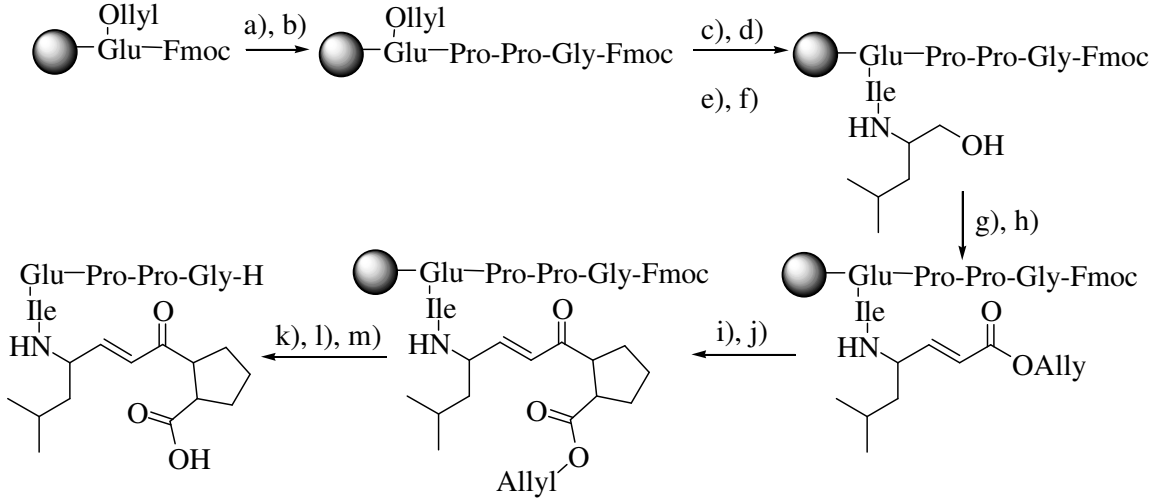
Chung *et al.* synthesized a series of diffucult sequences of tri to heptapeptide by microwave activation [64]. *N*-Fmoc-*L*-amino acid was activated by thionyl chloride and benzotriazole to obtain *N*-Fmoc-(a-aminoacyl)benzotriazoles **46** that had been used in the solid phase peptide synthesis on the rink amide solid support. The crude residue was obtained in 65-77% yields after cleavage and no evidence of any racemization or by product. A possible explanation can be impacted of microwave irradiation on the environment of the growing peptide [65]. The microwave irradiation can be reduced the forming of aggregates with itself or neighboring chains *via* hydrogen bonding of the peptide. The microwave energy also represents a fast and efficient way to enhance both the deprotection and hindered coupling reactions (scheme 33).



a) $SOCl_2$ (1.0 equiv), b) benzotriazole (4.0 equiv), c) N-α-aminoacyllbenzotriazole ***46****, DMF/CH_2Cl_2, microwave irradiation.*

Scheme 33: Peptide bond formation by using microwave activation.

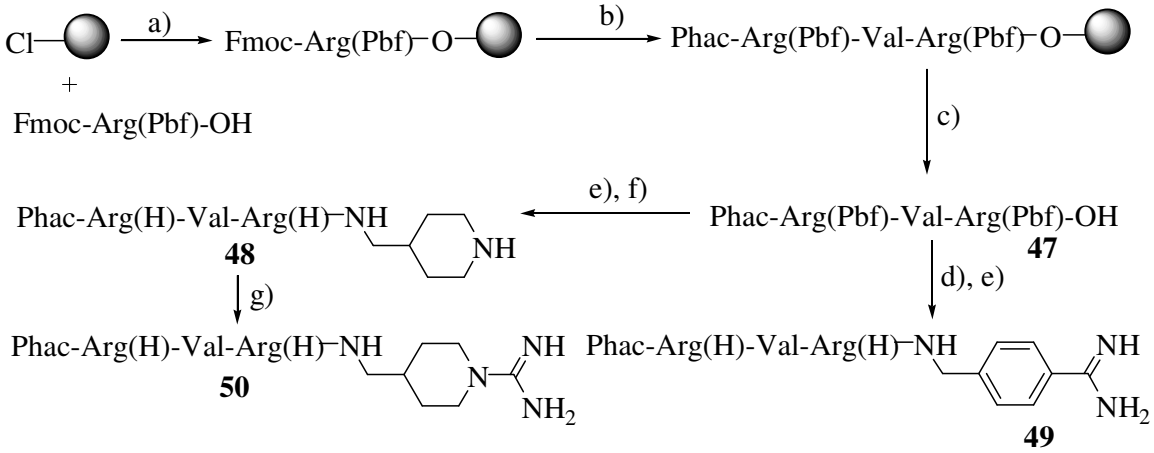
The application of solid phase peptide synthesis is done by using the Horner-Emmons reaction to generate olefin part in peptide backbone [66]. Akaji *et al.* reported the modification of solid phase procedure by using Dess-Martin periodinane to oxidize peptide alcohol and following by Horner-Emmons reaction (scheme 34) [67].



a) 20% piperidine/DMF, b) Fmoc-based SPPS, c) $[(C_6H_5)_3P]_4Pd(0)$, d) H-Ile-O-allyl/DCC/HOBt, e) $[(C_6H_5)_3P]_4Pd(0)$, f) H-leucinol/DCC/HOBt, g) Dess-Martin periodinane, h) $(EtO)_2P(O)CH_2COO$-allyl/$[(CH_3)_3Si]_2NLi$, i) $[(C_6H_5)_3P]_4Pd(0)$, j) H-Pro-O-allyl/HATU/HOAt, k) $[(C_6H_5)_3P]_4Pd(0)$, l) 20% piperidine/DMF, m) TFA/H_2O (95:5).

Scheme 34: Synthesis of olefin peptide.

Recently, the combination between solid and solution phase synthesis is a new approach to synthesize 4-amidinobenzylamide, 4-(amidino)piperidine and their analogues [68]. The linear peptide core (Arg(Pbf)-Val-Arg(Pbf)) was prepared by standard Fmoc SPPS on 2-chlorotrityl chloride resin in the presence of HOBt and HBTU for coupling reagent. The side chain protection was done in solution phase procedure. 4-amidinobenzylamine x HCl and *1H*-pyrazole-1-carboxamidine x HCl were coupled with peptide **47** and **48** in the presence of PyBOP/DIPEA and Cl-HOBt to provide compound **49** and **50**, respectively (scheme 35).



a) loading of 2-chlorotrityl chloride resin, Fmoc-Arg(Pbf)-OH, DIPEA (4.0 equiv), CH_2Cl_2, 2 h, b) Fmoc SPPS (HOBt, HBTU), c) 1% TFA in CH_2Cl_2, d) 4-amidinobenzylamine x HCl, PyBOP (1.1 equiv), 6-Cl-HOBt (3.0 equiv), DIPEA (3.0 equiv) in DMF, 2 h, e) TFA/TIS/H_2O (95/2.5/2.5, v/v/v), 2 h, f) 1-Boc-4-(aminomethyl)piperidine, PyBOP (1.1 equiv), 6-Cl-HOBt (3.0 equiv), DIPEA (3.0 equiv) in DMF, 2 h, g) 1H-pyrazole-1-carboxamidine x HCl (3.0 equiv), DIPEA (4.0 equiv) in DMF, 16 h.

Scheme 35: Synthesis of 4-amidinobenzylamide **47** and 4-(amidino)piperidine **48**.

1.3) AIMS OF THIS THESIS

This thesis is principally concerned with the synthesis of a range of peptides and their derivatives including structure activity relationship (SAR) study and biological assays of these compounds.

Chapter 1 obtains introduction of two main parts which is background of CDK4 inhibitor and briefly reviews about biological assays and peptides synthesis. Both solid and solution phase peptide synthesis are also reviewed in this chapter. Furthermore, application of peptide synthesis includes updates of coupling reagents and techniques of peptide synthesis are obtained in this chapter.

This chapter 2 provides discussion of solution phase peptide synthesis of protected hexapeptide **64** (*N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe) and attempts to synthesize parent hexapeptide **1** (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe) from compound **64** by side chain modification. In this chapter, we also discuss about an alternative route that is applied to our synthesis to obtain a series of linear hexapeptides with difference functional group at terminus. Finally, we also discuss about the synthesis of a series of *L*-alanine scanning compounds.

The parent hexapeptide **1**, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe is successfully synthesized by solid phase method. A series of alanine scanning compounds are synthesized using an analogous synthetic method. The results and discussion of peptide synthetic methods are described in chapter 3.

As we know that cyclic peptides have several advantages as drug candidates. Cyclization strategy has been proved to be very useful in developing therapeutically peptides agents. Since peptides generally adapt highly flexible conformations in solution, a cyclization approach has been commonly used to reduce the conformational freedom of these molecules and obtain hydrophobic structure. Therefore, chapter 4 includes interesting literature reviews of cyclic peptide synthesis, results and discussion and conclusion of cyclic peptide **161** and **168** synthesis.

In chapter 5, we report procedures and full characterizations for all peptides that are synthesized by solution, solid phase methods and macrocyclization.

Chapter 6 provides general techniques, material and method, cell lines and tissue culture conditions that are used in this project. The methods for cell based ELISA, cell proliferation and clonogenic assay are described in this chapter.

Chapter 7 provides details of biological assay development. A short term, cell based ELISA assay and cell proliferation assay are developed and used in this work because both of these assays requires small amount of synthetic peptides. Unfortunately, these assays do not show any significant results with our peptide. Therefore, clonogenic assay is developed although this assay needs long-term incubation (maximum 29 days) and requires a lot of synthetic peptide per each assay. The details for all assays development are discussed in this chapter.

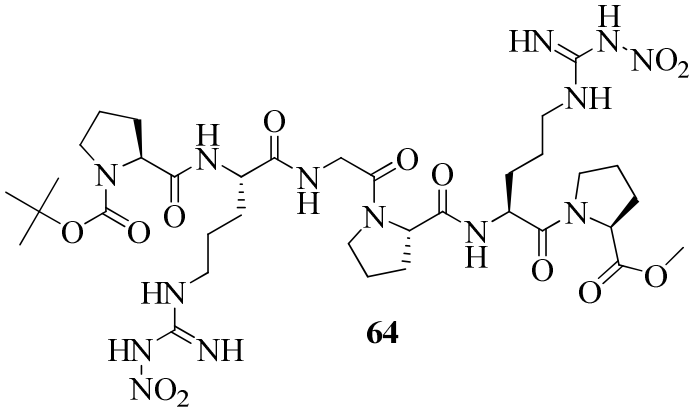
In chapter 8, we report structure activity relationship (SAR) study of linear and cyclic peptides which are synthesized by solid and solution phase method. The anticancer activities of peptides are studied by clonogenic assay and compared the results with THR1.

CHAPTER 2: RESULTS AND DISCUSSION

SOLUTION PHASE PEPTIDE SYNTHESIS OF *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64** AND ITS ANALOGUES.

2.1) SOLUTION PHASE PEPTIDE SYNTHESIS

Solution phase method is suitable for large scale peptide synthesis. Stepwise coupling is based on the sequential addition of an *N*-protected amino acid to the growing amino component of a peptide chain [69]. The objectives of this work were to develop the solution phase synthetic method and to synthesize protected hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64** (scheme 36) and its analogues for structure activity relationships (SARs) study.



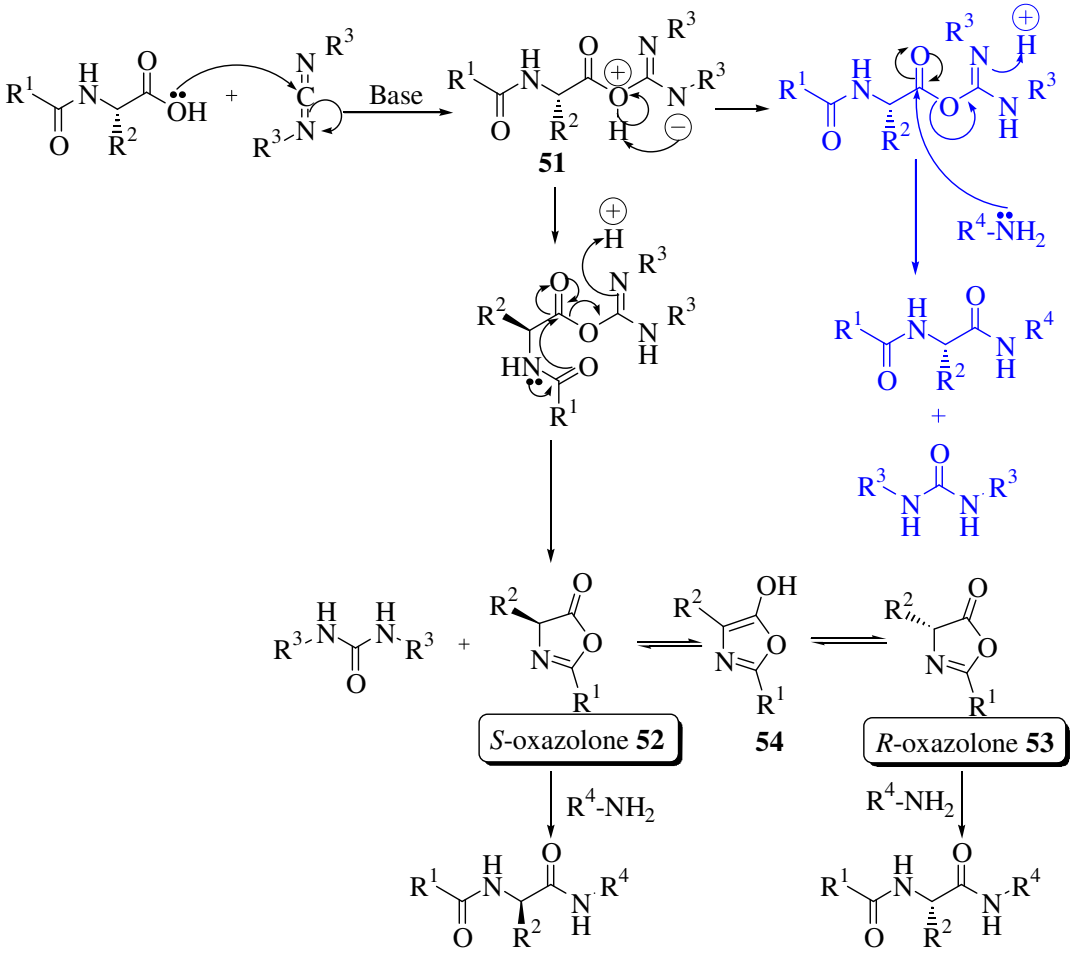
64

Scheme 36: Chemical structure of protected hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64**.

2.1.1) Coupling reaction

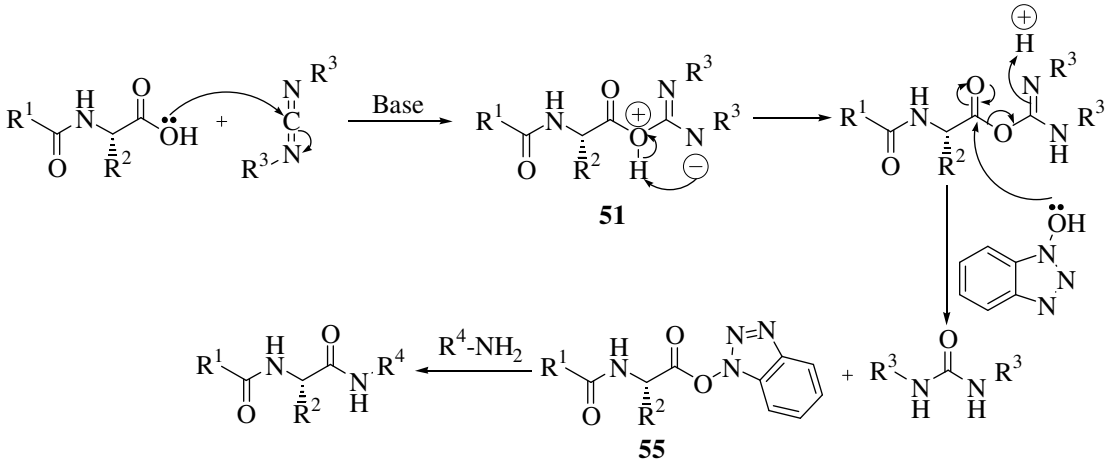
Peptide coupling is undertaken based on stepwise solution phase synthesis. A range of reagent can be used for activation of the *C*-terminus of amino acids in a peptide chain. Carbodiimides are chosen for this purpose as they are cheap and suitable for large scale solution phase peptide synthesis [70]. The first step in the coupling process is the nucleophilic attack of the carboxylic acid (COOH) on the carbodiimide carbon to give an *O*-acyl-isourea compound **A**. The carbodiimide activation method has a high propensity for racemization because of the high reactivity of the *O*-acyl-isourea **51**, which can undergo intramolecular cyclization and formation of an oxazolone **52**. This cyclic intermediate can

be easily racemized to give compound **53** *via* an aromatic intermediate **54** as shown in scheme 37 [71]. The racemization mechanism through the formation of oxazolone **53** and **54** has been extensively investigated [72].



Scheme 37: Coupling reaction through carbodiimide activation: mechanism of racemization *via* the formation of oxazolone.

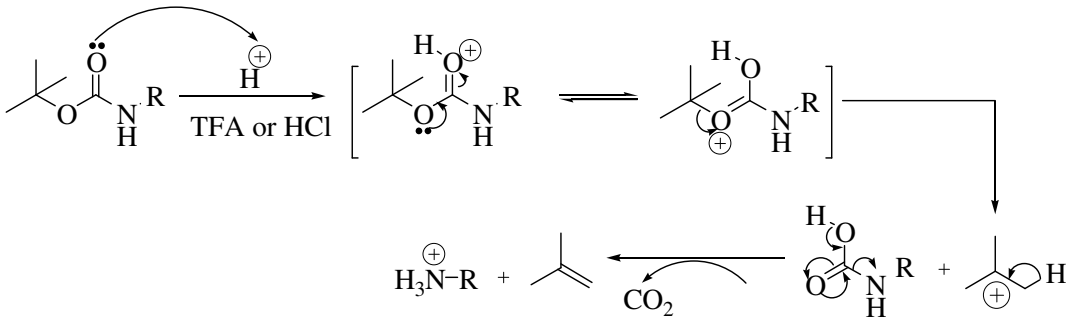
The addition of 1-hydroxybenzotriazole (HOBt) to the reaction mixture can be minimized the racemization by forming a less-reactive HOBt ester [73]. Derivative **55** is less reactive than **51**, but it is more stable and less prone to racemization. The mechanism is shown in scheme 38. Dicyclohexylurea (DCU) is formed as by product which can easily be filtered from the reaction mixture.



Scheme 38: Coupling reaction through carbodiimide and the addition of 1-hydroxybenzotriazole (HOBt).

2.1.2 *N*-Boc deprotection by strong acid.

Selective deprotection of a particular functional group in the presence of others is one of the most important transformations in organic synthesis [74]. *N*-Boc deprotection is undertaken by strong acid such as trifluoroacetic acid (TFA) in dichloromethane (CH_2Cl_2) [75] or HCl in dioxane [76] to obtain desired product in high yield and can be used in the next step without further purification (scheme 39)

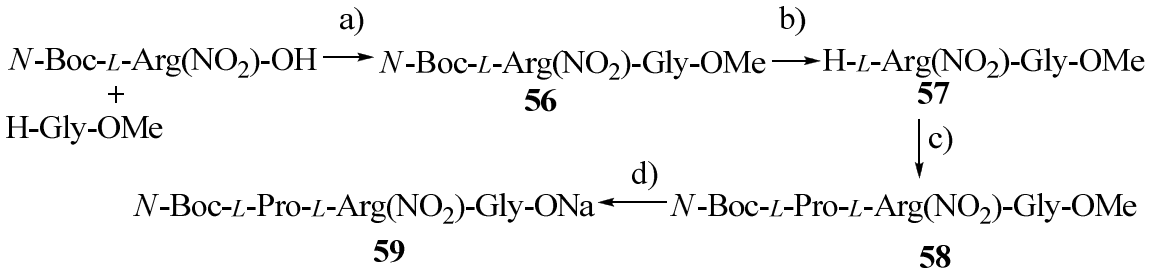


Scheme 39: *N*-Boc deprotection by strong acids.

2.2) SYNTHESIS OF *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 64.

The peptide address sequences were synthesized by the solution phase method using DCC in the presence of HOBt. The nitro group was used in the protection of the guanidine function of the *L*-arginine residue. Methyl ester was employed in protecting of the *C*-terminus of amino acids. Tripeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa, **59** was

synthesized according to the steps shown in scheme 40. Dipeptide **56** was obtained as a white solid in high yield from the coupling reaction between the commercially available *N*-Boc-*L*-Arg(NO_2)-OH and H-Gly-OMe. All solvents were removed *in vacuo*. The residue was extracted with CH_2Cl_2, small amount of cold water, cold 10% aq. citric acid and cold 10% aq. $NaHCO_3$. The crude residue was purified by column chromatography. The *N*-Boc deprotection of dipeptide **56** was done with 20% TFA in CH_2Cl_2, to provide free *N*-terminus compound **57** as a white solid in high yield and could be used in the next step without further purification. The tripeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-OMe, **58** was prepared under the same coupling conditions between commercially available *N*-Boc-*L*-Pro-OH and dipeptide **57** to give compound **58** as a white solid in moderate yield. Hydrolysis reaction was followed to remove *C*-terminus protecting group (-OMe) using 1.0 equiv of 1M aq. NaOH in ethanol, to provide tripeptide precursor **59** as a white solid in medium yield and could be used in the next step without further purification.

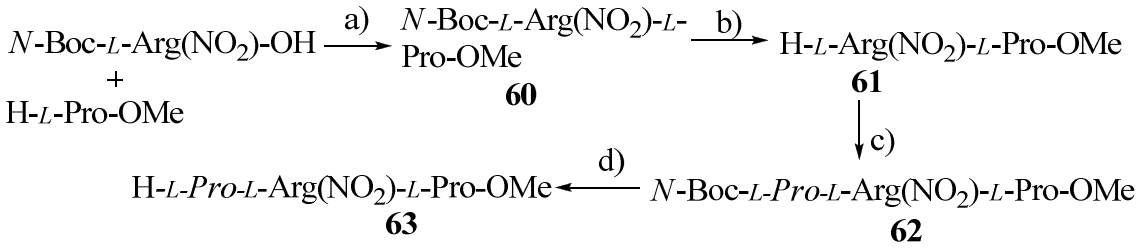


a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 78%, b) 20% TFA, CH_2Cl_2, 83%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 67%, d) 1 M NaOH (aq. preparation, 1.0 equiv), EtOH, 53%.

Scheme 40: Synthesis of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa **59** precursor.

Tripeptide, H-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **63** was synthesized according to the steps shown in scheme 41. Dipeptide, *N*-Boc-*L*-Arg(NO_2)-*L*-Pro-OMe, **60** was prepared under the same coupling conditions between commercially available *N*-Boc-*L*-Arg(NO_2)-OH and H-*L*-Pro-OMe. Compound **60** was obtained as a white solid in moderate yield. *N*-Boc deprotection of compound **60** was undertaken with 20% TFA in CH_2Cl_2 to provide free *N*-terminus compound **61** as a white solid in high yield. The peptide, H-*L*-Arg(NO_2)-*L*-Pro-OMe, **61** could be used in the next step without further purification. Tripeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **62** was prepared under the same coupling conditions between

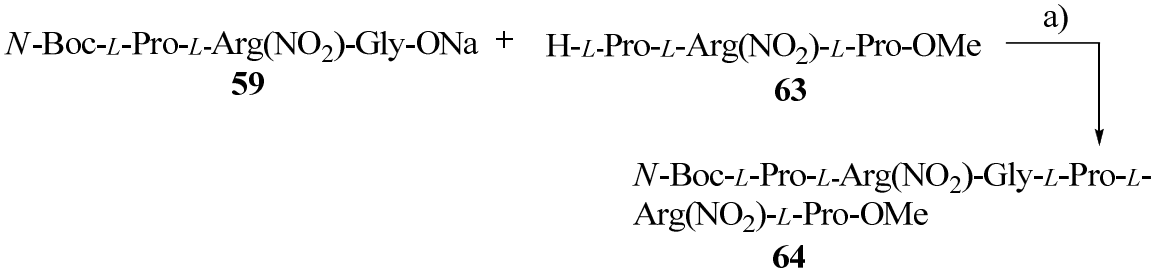
commercially available *N*-Boc-*L*-Pro-OH and dipeptide **61** to give compound **62** as a white solid in moderate yield. The *N*-Boc deprotection of compound **62**, under the same conditions as previously, provided tripeptide precursor **63** as a white solid in high yield.



a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 60%, b) 20% TFA, CH_2Cl_2, 85%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 60%, d) 20% TFA, CH_2Cl_2, 80%.

Scheme 41: Synthesis of H-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **63** precursor.

Finally, hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **64** was synthesized according to the steps shown in scheme 42 by the coupling under the same conditions between the tripeptide precursors **59** and **63**. The compound **64** was obtained as a white solid in medium yield.



a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 62%.

Scheme 42: Synthesis of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64**.

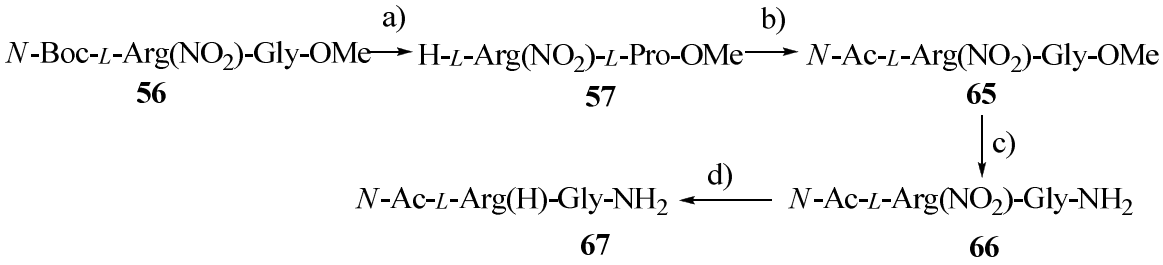
2.3) MODEL STUDY OF SIDE CHAIN MODIFICATION *L*-Arg(NO_2)-Gly SERIES.

The objective of this work was to synthesize a target hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, **1** (scheme 2) for biological assay by side chain modification of hexapeptide **64** *via N*-Boc deprotection, *N*-terminus acetylation, *C*-terminus

amide formation and catalytic hydrogenolysis to remove nitro protecting group from guanidine side chain of arginine (Arg), respectively.

Before we started to synthesize the target hexapeptide **1** from compound **64**, we had synthesized a model compound **67** from *L*-Arg(NO_2)-Gly series. The simple dipeptide, *N*-Boc-*L*-Arg(NO_2)-Gly-OMe, **56** was chosen as starting compound for model study because dipeptide **56** was very easy to synthesize by general solution phase coupling reaction and obtained in high yield after column chromatography. According to scheme 43, compound **67**, *N*-Ac-*L*-Arg(H)-Gly-NH_2, was obtained in high yield *via* a four-step procedure involving *N*-Boc deprotection, *N*-acetylation, amide formation and catalytic hydrogenolysis.

N-Boc deprotection of dipeptide **56** by 4M HCl in dioxane resulted in compound **57** as a white solid in very high yield and was used in the next step without further purification. The acetylation of dipeptide **57** was catalyzed by DMAP using acetic anhydride as the acetylating agent to afford compound **65** in high yield. In the next step, *C*-terminus of *N*-Ac-*L*-Arg(NO_2)-Gly-OMe **65** was modified by amide formation with saturated NH_3 in MeOH which was prepared by bubbling ammonia gas through MeOH for 2 h. The peptide **65** was treated with sat. NH_3 in MeOH at 0 ºC for 3 h. The solution mixture was warmed up to room temperature and stirred overnight. All solvents were removed *in vacuo* and desired product **66** was obtained as pale yellow oil. As result, there was some starting material **65** left which could not be isolated by normal phase column chromatography. To avoid this problem and get better yield, we used 35% ammonium hydroxide (ammonia in water) instead of sat. NH_3 in MeOH. The reaction mixture was stirred at 0 ºC for 3 h and warmed to room temperature overnight. All solvents were removed *in vacuo*. The desired product was precipitated by MeOH and diethyl ether to obtain amide product **66** as a white solid in excellent yield. Finally, nitro protecting group of peptide **66** was removed by catalytic hydrogenolysis in the presence of Pd/C. The reaction mixture was stirred overnight, filtered through celite pad. All solvents were removed *in vacuo*. The desired product **67** was obtained as a white solid in high yield.

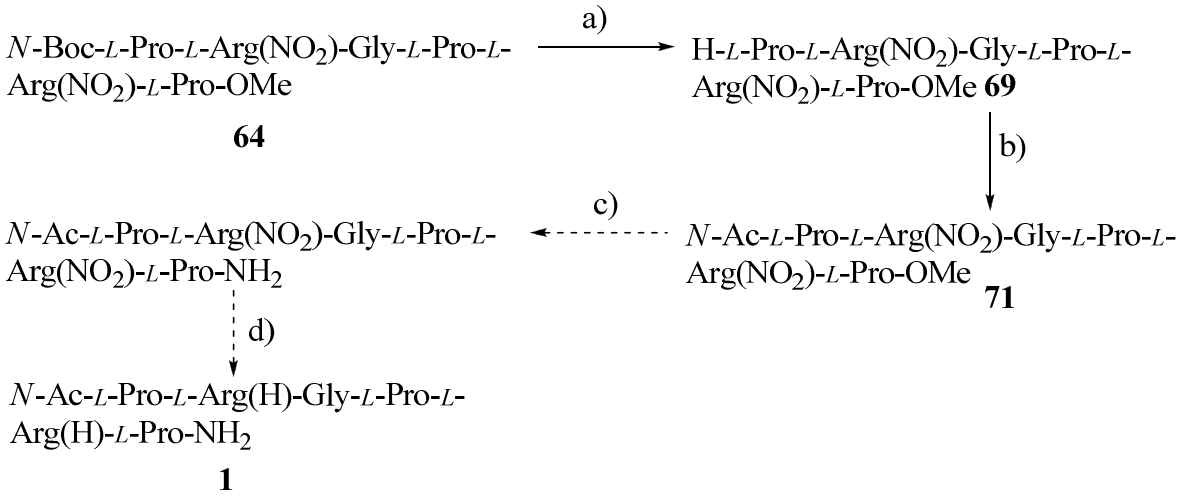


a) 4 M HCl/dioxane (3.0 equiv), 85%, b) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight, 62%, c) 35% NH_3 in H_2O, 95%, d) H_2, Pd/C, MeOH/AcOH (3:1), 78%.

Scheme 43: Synthesis of *N*-Ac-*L*-Arg(H)-Gly-NH_2 **67**.

2.4) STRUCTURAL MODIFICATION OF HEXAPEPTIDE, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, 64 FOR STRUCTURE ACTIVITY RELATIONSHIP (SAR) STUDY.

After we successfully synthesized model compound, *N*-Ac-*L*-Arg(H)-Gly-NH_2, **67**, we proceeded with the synthesis of the target hexapeptide **1**, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, using an analogous synthetic procedure. Attempted side chain modification of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64** is shown in scheme 44. *N*-Boc deprotection of hexapeptide **64** by 4M HCl in dioxane offered free *N*-terminus peptide as a white solid in high yield. This was then followed by *N*-terminus acetylation of peptide **69** which was catalyzed by DMAP using Ac_2O as the acetylating agent to afford compound **71** as a white solid in medium yield. *N*-acetyl peptide **71** was treated with 35% NH_4OH (NH_3 in water) at 0 °C for 3 h and warmed up to room temperature overnight. All solvents were removed *in vacuo*. The crude residue was obtained as pale yellow oil which could not be purified by normal phase column chromatography because of the high polarity of the compound. The crude residue was then precipitated from MeOH/ Et_2O to obtain *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-NH_2 as a pale yellow solid in medium yield. As a result, proton peaks were so broad, complicated and could not be identified or interpreted because there were many nitrogen and oxygen atoms in the peptide chain which could form hydrogen bonding with water in NMR solvent (d^6-DMSO). We also changed NMR solvent to d^4-MeOD but the results of ^{1}H-NMR spectrum were the same as previously described. LRMS (ES^+) spectrum was also complicated and we could not find any ionization peaks of molecular ion.



a) 4 M HCl/dioxane (3.0 equiv), 96%, b) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight, 60%, c) 35% NH_3 in H_2O, d) H_2, Pd/C, MeOH/AcOH (3:1).

Scheme 44: An attempt to synthesis the parent hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, **1**.

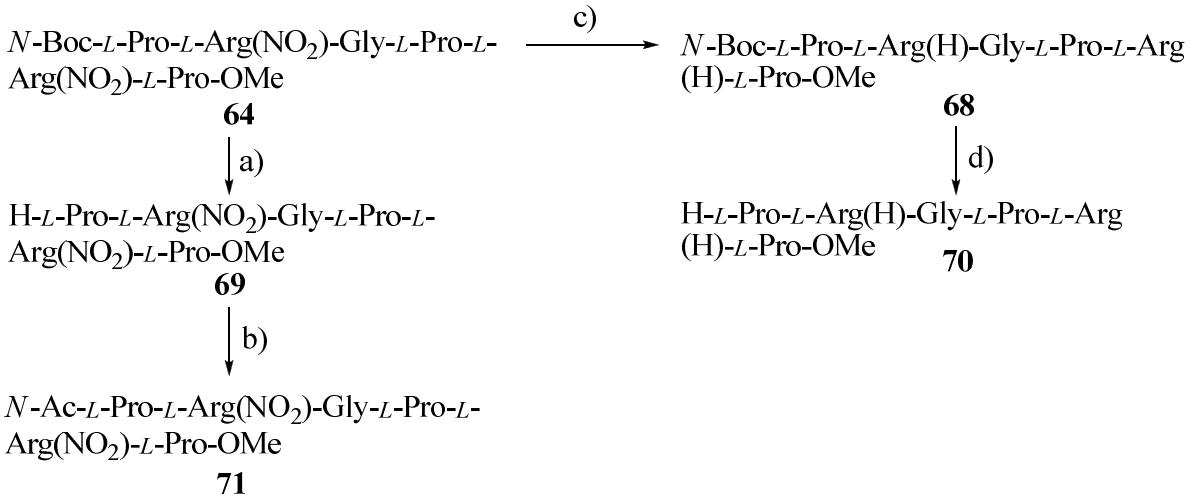
For the above mentioned reasons, we could not successfully to synthesize target hexapeptide **1**. Therefore, an alternative analogous of side chain modification of compound **64** were designed and synthesized for structure activity relationship (SAR) study. The objective of this work was to investigate which functional group at side chain and *N*- or *C*-terminus of peptide had the most potent for anticancer activity (scheme 45).

Firstly, nitro protecting groups (-NO_2) from arginine side chain were removed from guanidine parts of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64** by catalytic hydrogenolysis in the presence of Pd/C**.** The desired product was precipitated to provide *N*-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe **68** as white solid in high yield.

Secondly, *N*-Boc group was removed from hexapeptide **64** by 4M HCl in dioxane to obtain H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe x HCl **69** as a white solid in high yield.

Then, H-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe x HCl **70** was synthesized by *N*-Boc deprotection of compound **68** with 4 M HCl in dioxane as a white solid in high yield.

Finally, compound **69** was catalyzed by DMAP using Ac_2O as an acetylating agent to afford *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **71** as a white solid in moderate yield.



a) 4 M HCl/dioxane (3.0 equiv), 95%, b) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight, 63%, c) H_2, Pd/C, MeOH/AcOH (3:1), 92%, d) 4 M HCl/dioxane (3.0 equiv), 98%.

Scheme 45: Side chain modification of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **64**.

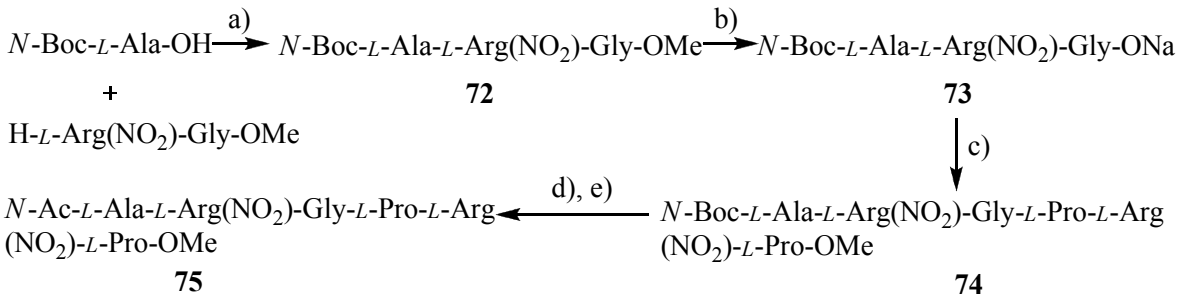
In conclusion, hexapeptides, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe**, 64** was successfully synthesized by solution phase method. A series of partially side chain modification of compound **64** had been developed and synthesized for SAR study. The biological assays are described in chapter 8, we found that *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **71** displayed an excellent anticancer activity against RT112 bladder cancer cells without killing normal fibroblast cell lines. The structural characterization was done by NMR (^1H and ^{13}C) spectra to confirm structure of peptides. LRMS and HRMS were done to confirm mass of the peptides. Especially, single chromatogram of HPLC was achieved to confirm purity of the final hexapeptides.

2.5) SOLUTION PHASE PEPTIDE SYNTHESIS OF *L*-ALANINE (Ala) SCANNING PEPTIDES

The objectives of this work were to synthesize a series of compound **71** by replacing each amino acid residue with *L*-alanine using an analogous synthetic procedure and to investigate which amino acid residue had shown the most anticancer activity by comparing with hexapeptide **71**. Amino acid replacement by alanine or alanine scanning method is a common strategy for testing the role of each amino acid residue in peptide chain to anticancer activity [74]. Alanine is used because it has a nonfunctional hydrophobic side chain and generally preserves the backbone conformation of the peptides.

2.5.1) SYNTHESIS OF *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 76.

Hexapeptide, *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **76** was synthesized according to the steps shown in scheme 46. Tripeptide **72** was obtained as a white solid in high yield from the coupling reaction between the commercially available *N*-Boc-*L*-Ala-OH and H-*L*-Arg(NO_2)-Gly-OMe x TFA **57** (scheme 40). Hydrolysis reaction was followed to remove *C*-terminus protecting group (OMe) by using 1 equiv of 1M aq. NaOH in ethanol. The desired product was then precipitated and could be used in the next step without further purification to obtain tripeptide precursor, *N*-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-ONa, **73** as a white solid in high yield. Hexapeptide, *N*-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **74** could be synthesized from the coupling under same conditions between tripeptide precursors *N*-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-ONa **73** and H-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **63** (scheme 41) to obtain compound **74** as a white solid in moderate yield which had the amino acid in the first position of the peptide chain replaced with *L*-alanine. *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **74** was synthesized by *N*-Boc deprotection of compound **74** with 4M HCl in dioxane to obtain H-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **75** as a white solid in high yield. Acetylation of hexapeptide **75** was catalyzed by DMAP using Ac_2O as the acetylationg agent. The desired product was purified by column chromatography and precipitated to obtain *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **76** as a white solid in medium yield.

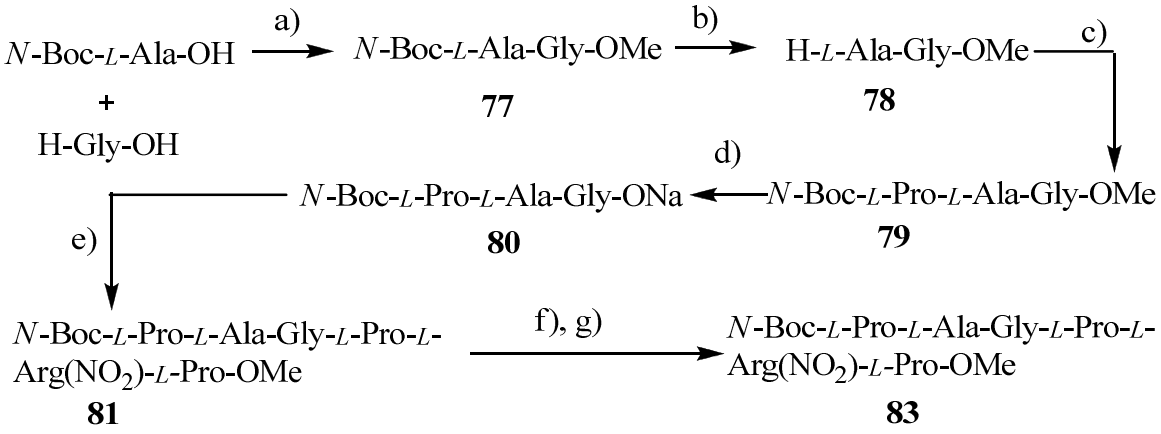


a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 70%, b) 1 M NaOH (aq. preparation, 1.0 equiv), EtOH, 75%, c) H-L-Pro-L-Arg(NO$_2$)-L-Pro-OMe ***63*** *(1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, overnight, 54%, d) 4 M HCl/dioxane (3.0 equiv), 95%, e) Ac$_2$O (5.0 equiv), NEt$_3$ (5.0 equiv), DMF, RT, overnight, 65%.*

Scheme 46: Synthesis of *N*-Ac-*L*-Ala-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **76**.

2.5.2) SYNTHESIS OF *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 83.

Hexapeptide, *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **83** was synthesized using an analogous synthetic procedure (scheme 47). *N*-Boc-*L*-Ala-Gly-OMe **77** and *N*-Boc-*L*-Pro-*L*-Ala-Gly-OMe **79** could be synthesized from coupling reaction under general conditions in low yield. Because there were a lot of products in crude residue and then after purified by column chromatography, the product was obtained as colorless oil in low % yield. Hydrolysis reaction was followed to remove *C*-terminus protecting group (OMe) using 1 equiv of 1M aq. NaOH in ethanol. The tripeptide precursor, *N*-Boc-*L*-Pro-*L*-Ala-Gly-ONa**, 80** was obtained as colorless oil in high yield and then coupled with H-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **63** (scheme 41) under the same conditions to provide *N*-Boc-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **81** as a white solid in high yield which had the amino acid in the second position of the peptide chain replaced with *L*-alanine. All products in this scheme were UV inactive compounds then we used ninhydrin stain for TLC monitoring. *N*-Ac-peptide **83** was synthesized using an analogous synthetic procedure *via* *N*-Boc deprotection by 4M HCl/dioxane and *N*-acetylation by Ac$_2$O. The desired product was purified by column chromatography and precipitated to obtain *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **83** as a white solid in medium yield.



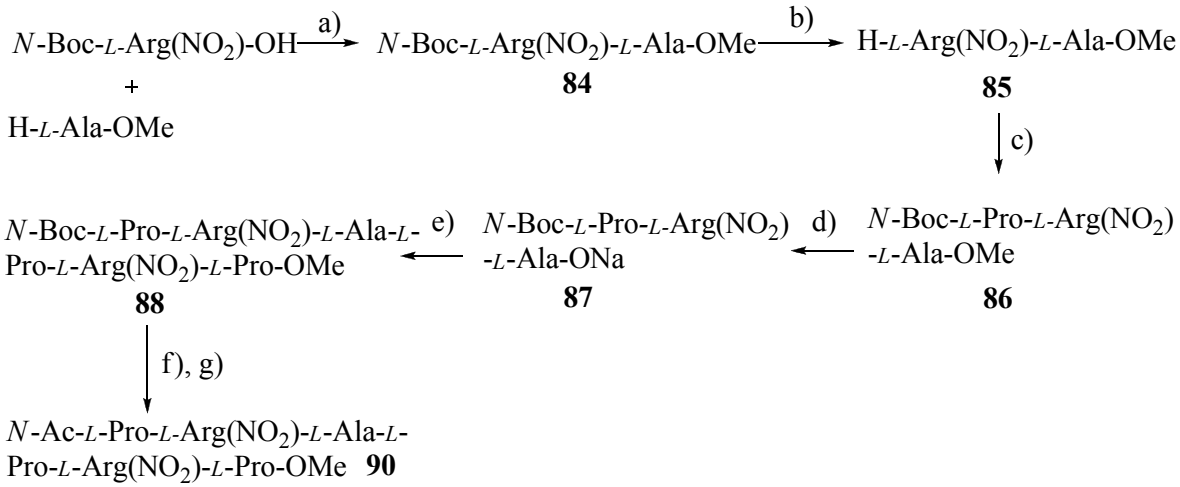
a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 42%, b) 20% TFA/CH_2Cl_2 (3.0 equiv), 89%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 38%, d) 1 M NaOH (aq. preparation, 1.0 equiv), EtOH, 95%, e) H-L-Pro-L-Arg(NO_2)-L-Pro-OMe ***63*** *(1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, overnight, 80%, f) 4 M HCl/dioxane (3.0 equiv), 97%, g) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight 58%.*

Scheme 47: Synthesis of *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **83**.

2.5.3) SYNTHESIS OF *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 90.

Hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **90** was synthesized according to the steps shown in scheme 48. Dipeptide, *N*-Boc-*L*-Arg(NO_2)-*L*-Ala-OMe, **84** was obtained in high yield by general coupling between commercially available *N*-Boc-*L*-Arg(NO_2)-OH and H-*L*-Ala-OMe. *N*-Boc deprotection of the dipeptide **84** was done by 20% TFA in CH_2Cl_2 and followed by coupling reaction under the same conditions with *N*-Boc-*L*-Pro-OH. The crude residue was purified by column chromatography and precipitated to obtain tripeptide **86** as a white solid in moderate yield. Hydrolysis reaction was followed to remove *C*-terminus protecting group (OMe) using 1 equiv of 1M aq. NaOH in ethanol. A tripeptide precursor, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-ONa**,** **87** was obtained as colorless oil in high yield and then coupled with H-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **63** (scheme 41) under the same conditions to provide *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **88** as a white solid in high yield. *N*-Ac-

peptide **90** was synthesized using an analogous synthetic procedure and obtained as a white solid in moderate yield which had the amino acid in the third position of the peptide chain replaced with *L*-alanine (scheme 48).



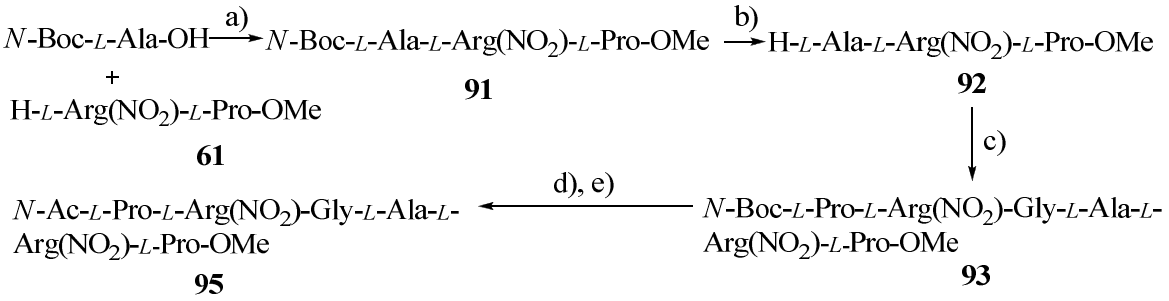
a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 70%, b) 20% TFA/CH_2Cl_2 (3.0 equiv), 87%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 65%, d) 1 M NaOH (aq. preparation, 1.0 equiv), EtOH, 74%, e) H-L-Pro-L-Arg(NO_2)-L-Pro-OMe ***63*** *(1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, overnight, 60%, f) 4 M HCl/dioxane (3.0 equiv), 92%, g) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight, 77%.*

Scheme 48: Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **90**.

2.5.4) SYNTHESIS OF *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe 95.

Hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **95**, was synthesized according to the steps shown in scheme 49. *N*-Boc-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **91** was prepared from general coupling reaction between commercially available *N*-Boc-*L*-Ala-OH and dipeptide **61** (scheme 41) under the same conditions. *N*-Boc deprotection of tripeptide **91** was done by 20% TFA in CH_2Cl_2 to obtain tripeptide precursor, H-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **92**. The desired product was obtained as a white solid and could be coupled with *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa **59** (scheme 40) to provide hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe, **93** as

a white solid in medium yield. *N*-Ac-peptide **95** was synthesized using an analogous synthetic procedure (2.5.1) and obtained as a white solid in moderate yield which had the amino acid in the forth position of the peptide chain replaced with *L*-alanine.



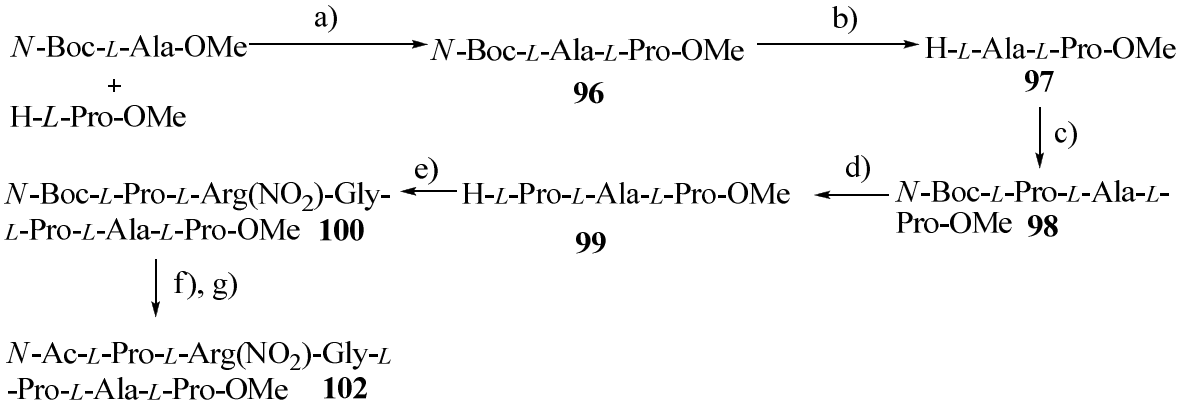
a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 58%, b) 20% TFA/CH_2Cl_2 (3.0 equiv), 79%, c) N-Boc-L-Pro-L-Arg(NO_2)-Gly-ONa ***59*** *(1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, overnight, 62%, d) 4 M HCl/dioxane (3.0 equiv), 97%, e)* Ac_2O *(5.0 equiv),* NEt_3 *(5.0 equiv), DMF, RT, overnight, 65%.*

Scheme 49: Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **95**.

2.5.5) SYNTHESIS OF *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe 102.

Hexapeptide, *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **102** was synthesized according to the steps shown in scheme 50. *N*-Boc-*L*-Ala-*L*-Pro-OMe **96** was synthesized from coupling reaction under the same conditions between commercially available *N*-Boc-*L*-Ala-OH and H-*L*-Pro-OMe. The crude residue was purified by column chromatography and obtained dipeptide 96 as a pale yellow oil in low yield. *N*-Boc deprotection of compound **96** was done by 20% TFA in CH_2Cl_2 and provided free *N*-terminus peptide **97** that could be used in the next step without further purification. *N*-Boc-*L*-Pro-*L*-Ala-*L*-Pro-OMe **98** could be synthesized from coupling reaction between *N*-Boc-*L*-Pro-OH and dipeptide **97** under general conditions in low yield. Both of peptides **96** and **98** were obtained in low yield because there were a lot of side products in crude residue and then after purified by column chromatography, the target product was obtained in low % yield. Tripeptide precursor, H-*L*-Pro-*L*-Ala-*L*-Pro-OMe, **99** was prepared by *N*-Boc deprotection of compound **98** by 20% TFA in CH_2Cl_2. The desired product was obtained as pale yellow oil in high yield and could be used in the next step without further purification. Hexapeptide,

N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe, **100** was obtained in medium yield by general coupling under the same conditions between tripeptide precursor **99** and *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa **43** (scheme 40). *N*-Ac-peptide **102** was synthesized using an analogous synthetic procedure (2.5.1) and obtained as a white solid in moderate yield which had the amino acid in the fifth position of the peptide chain replaced with alanine.

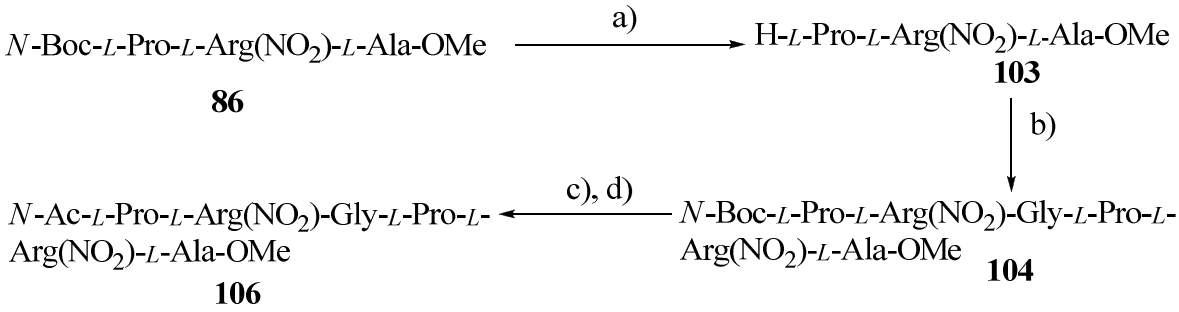


*a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 47%, b) 20% TFA/CH_2Cl_2 (3.0 equiv), 86%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, overnight, 43%, d) 20% TFA/CH_2Cl_2 (3.0 equiv), 87%, e) N-Boc-L-Pro-L-Arg(NO_2)-Gly-ONa **59** (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, overnight, 51%, f) 4 M HCl/dioxane (3.0 equiv), 92%, g) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight, 62%.*

Scheme 50: Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe **102**.

2.5.6) SYNTHESIS OF *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe 106.

N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe **106**, was synthesized according to the steps shown in scheme 51. Tripeptide precursor, H-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe, **103** was synthesized from *N*-Boc removal of tripeptide **86** (scheme 48) and then coupled with *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa **43** (scheme 40) under the same conditions to provide hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe, **104** as a white solid in medium yield. *N*-Ac-peptide **106** was synthesized using an analogous synthetic procedure (2.5.1) and obtained as a white solid in moderate yield which had the amino acid in the sixth position of the peptide chain replaced with *L*-alanine.



a) 20% TFA/CH_2Cl_2 (3.0 equiv), 89%, b) N-Boc-L-Pro-L-Arg(NO_2)-Gly-ONa ***59*** *(1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, overnight, 48%, c) 4 M HCl/dioxane (3.0 equiv), 95%, d) Ac_2O (5.0 equiv), NEt_3 (5.0 equiv), DMF, RT, overnight, 95%.*

Scheme 51: Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe **106**.

In conclusion, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **71** was a potent selective compound that showed an excellent anticancer activity against RT112 bladder cells. A series of *L*-alanine scanning compounds of **71** had been synthesized by an analogous synthetic method to obtain *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg (NO_2)-*L*-Pro-OMe **76**, *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **83,** *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **90**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **95**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe **102** and *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe **106**. The details of biological assays are explained in chapter 8. The structural characterization was done by NMR (^{1}H and ^{13}C) spectra to confirm structure of peptides. LRMS and HRMS were done to confirm mass of the peptides. Especially, single chromatogram of HPLC was achieved to confirm purity of the final hexapeptides (appendix 3).

CHAPTER 3: RESULTS AND DISCUSSION

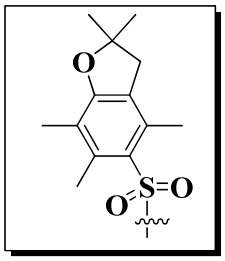
SOLID PHASE PEPTIDE SYNTHESIS OF *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **1** AND ITS ANALOGUES

3.1) SOLID PHASE PEPTIDE SYNTHESIS (SPPS)

The advent of solid-phase peptide synthesis (SPPS) has led to dramatic developments in peptide chemistry and related fields. Since Merrifield's pioneering work on SPPS in the 1960s, peptide preparation on a small to medium scale has almost exclusively been synthesized on solid supports [61]. According to chapter 2, we relied on a solution phase peptide synthesis to prepare hexapeptide **64** and its analogues. Attempts to synthesize target peptide **1**, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, by side chain modification of compound **64** were failed. In order to make our approach applicable for the synthesis of hexapeptide **1** (scheme 2), we developed a solid phase approach using the Fmoc-based methodology [78].

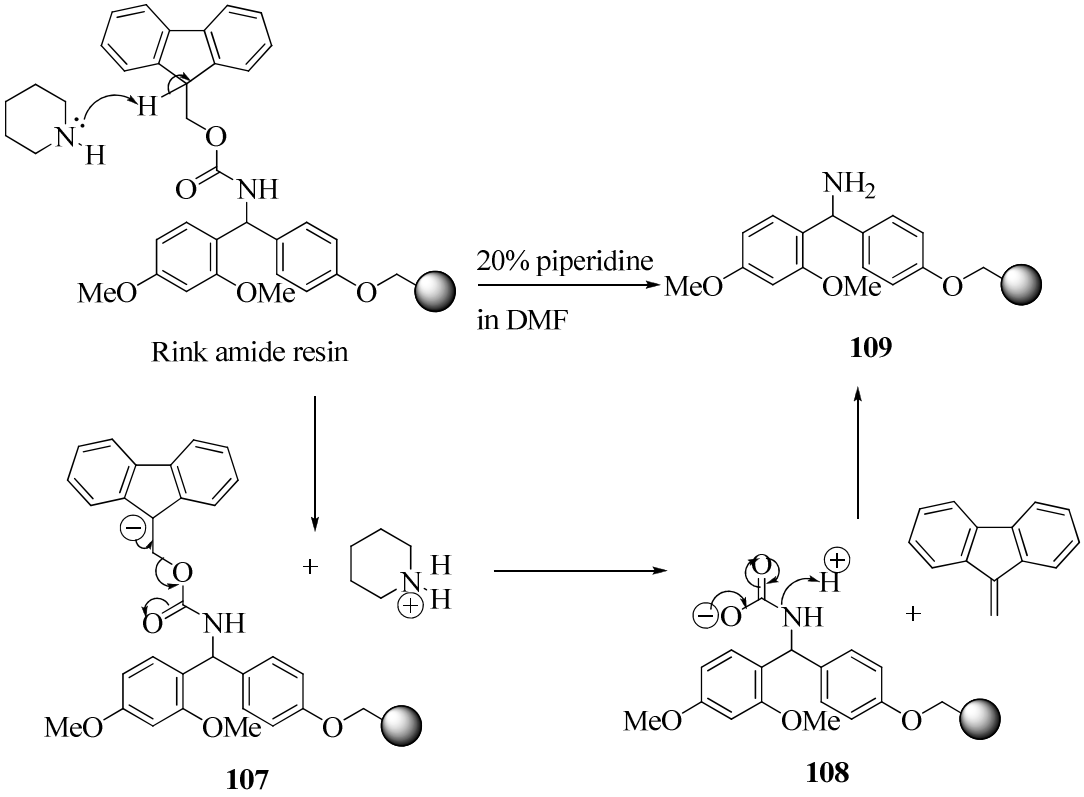
3.1.1) Materials and methods

a) Fmoc-L-amino acids: Linear peptides were synthesized using manual solid phase techniques employing the Fmoc protection scheme. All amino acids that used in this work were Fmoc-*L*-amino acid building blocks. A guanidine side chain of Fmoc-*L*-arginine residue is protected with Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) group which can be removed by TFA and thus is compatible with Fmoc strategy [79] (scheme 52).



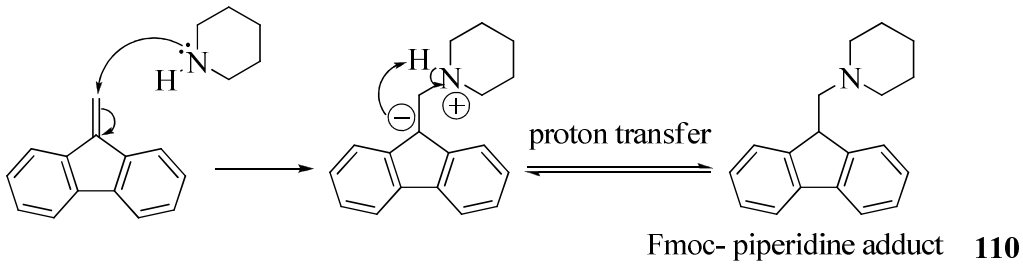
Scheme 52: Pbf protecting group of guanidine side chain of *L*-arginine (Arg) residue.

b) The Rink amide resin [80] is used as a solid support in order to produce *C*-terminus amide (CO-NH_2). This resin is first treated with 20% piperidine in DMF to remove Fmoc protecting group. The mechanism is shown in scheme 53 [81]. Piperidine is acted as a nucleophilic base and deprotonated to intermediate **107** and then decomposed to compound **108**. Carbon dioxide lost from carbamide **108** and yielded the free amine **109**.



Scheme 53: Fmoc deprotection from Rink amide resin by piperidine/DMF.

Piperidine is also acted as a scavenger to obtained methylene fluorine compound **110** as a by-product (scheme 54).



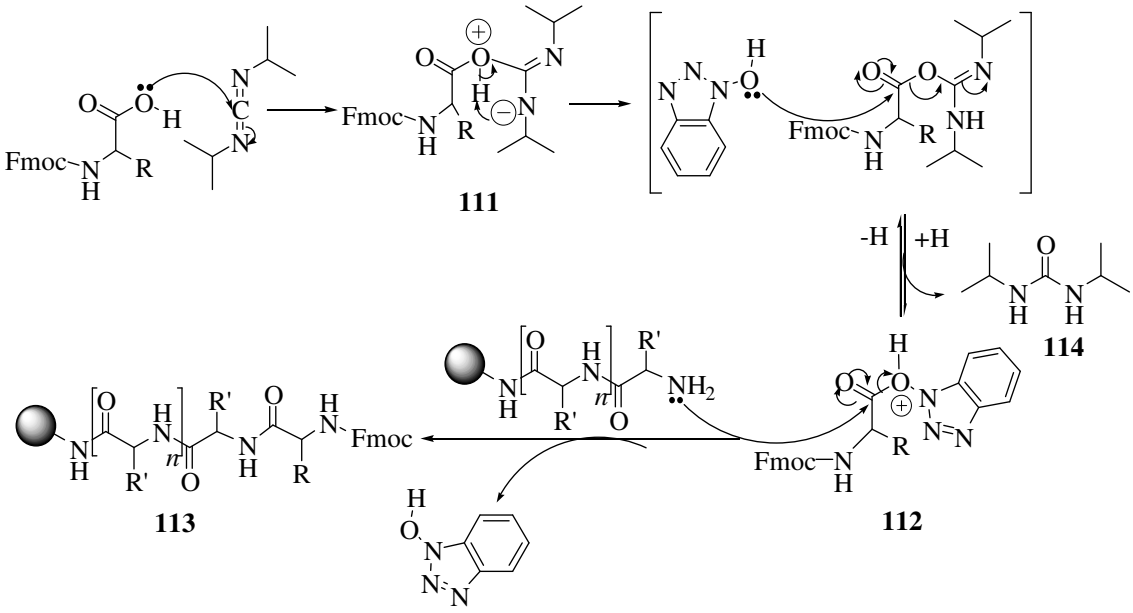
Scheme 54: Side reaction of Fmoc deprotection by piperidine.

c) Quantitative ninhydrin test is a useful method to determine *N*-free amino group, measure the total mmol of resin loading (by calculation) and monitor the progress of reaction [82].

This method involves the reaction of the free amine with ninhydin and determines the resulting chromophore in solution at 570 nm. All of the blue chromophore of the ninhydrin reaction with primary amines is found in the solution and the beads of the polystyrene support were colourless.

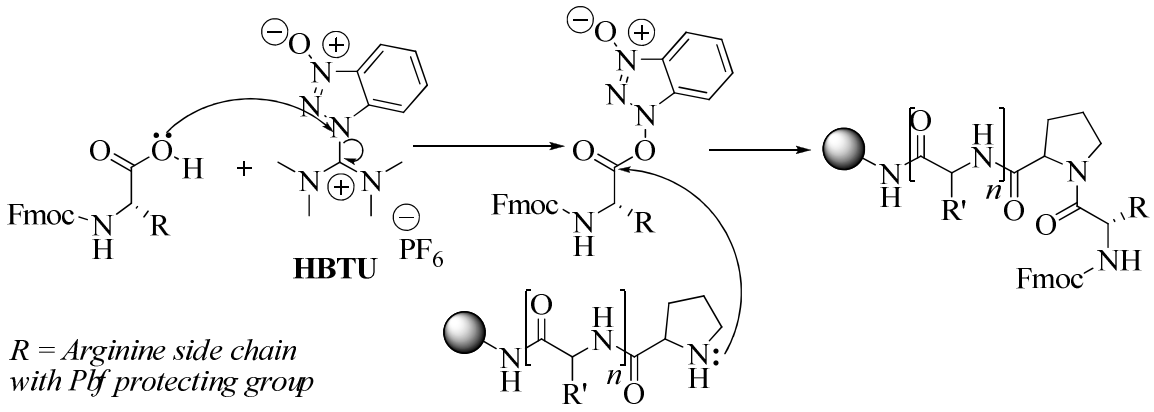
3.1.2) Peptide synthesis

Couplings were mediated with either DIC/HOBt or HBTU/HOBt in the presence of DIPEA in DMF. Fmoc removal was accomplished with two treatments of 20% piperidine in DMF. The mechanism of coupling amino acids with DIC and HOBt is shown in scheme 55 [83]. The first step in the coupling process is the nucleophilic attack of a carboxylic acid (COOH) on the carbodiimide carbon to give an *O*-acyl-isourea compound **111**. The addition of HOBt to the reaction mixture minimizes the racemization by forming a stable HOBt ester **112**. Nucleophilic attach of lone pair electrons from amino acid residue is reacted with intermediate **112** to provide product **113**. Urea **114** is found to be formed as by product which can easily be filtered from the reaction mixture.



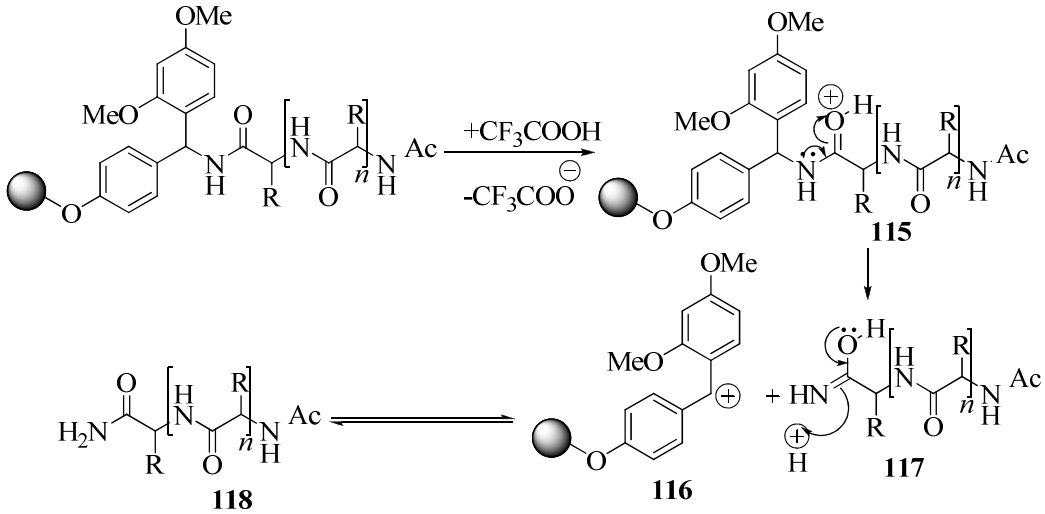
Scheme 55: Coupling reaction under normal conditions.

In case of the coupling reaction between Fmoc-*L*-Arg(Pbf)-OH and inert *L*-proline residue, we need more active coupling conditions such as HBTU and HOBt in the presence of DIPEA in DMF [84]. The mechanism is shown in scheme 56.



Scheme 56: coupling reaction with HBTU.

The peptides were *N*-terminally acetylated with acetic anhydride. Standard procedure of TFA/TIS/H_2O (96:2:2) was used for cleavage of peptide from resin and removal of side-chain protecting group [85]. The crude residues were purified by reverse phase flash column chromatography. A mechanism for the cleavage of a peptide from a Rink amide resin is shown in scheme 57. TFA can be protonated to compound **115** which is decayed easily into the carbocation **116**. Compound **116** can tautomerize to compound **117** very easily and then tautomerize to amide **118**.

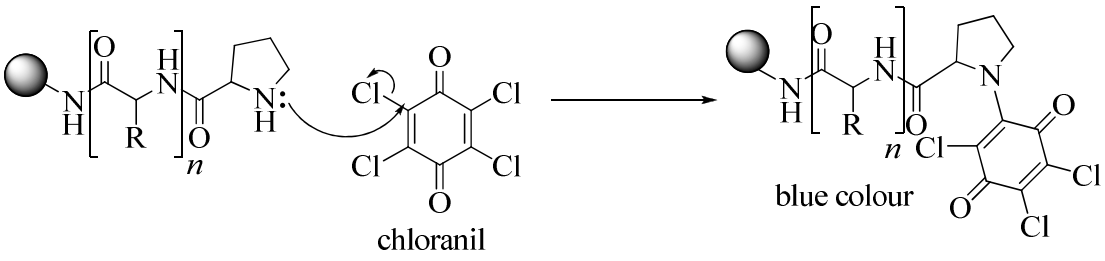


Scheme 57: Cleavage of peptide from rink amide resin.

3.1.3) Resin test

Since 1970, the Kaiser test had been used extensively to monitor the progress of amide bond formation during solid phase peptide synthesis [86]. This test is based on the reaction

of ninhydrin with primary amino group. In the presence of free primary amino group, the resin turns to deep blue colour for positive result. Nevertheless, the completeness of coupling reaction of primary amino acid residue is revealed as a pale yellow solution and colourless resin beads. The mechanism of primary *L*-amino acid residue with ninhydrin is shown in appendix 1.1. The presence of secondary amino group (such as proline), 2,3,5,6-tetrachlorobenzoquinone (chloranil) [87] had been used to monitor the progress amide bond formation and Fmoc deprotection. In the presence of free secondary amino group, the resin turns to deep blue colour for positive result. Nevertheless, the completeness of coupling reaction of secondary amino acid residue was revealed as a pale yellow solution and colourless resin beads. The reaction of chloranil and free *N*-terminus of *L*-proline residue is shown in scheme 58.

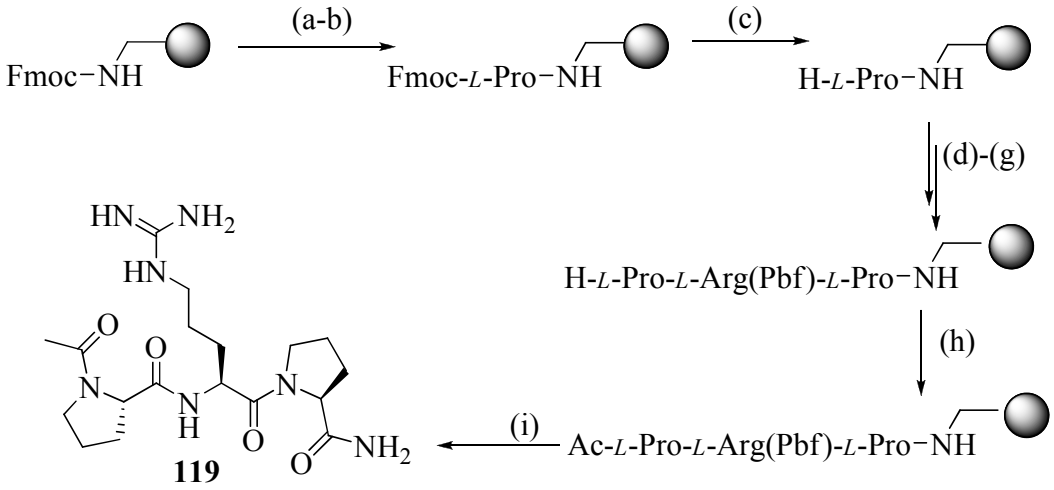


Scheme 58: The resins test between chloranil and secondary amino residue.

3.2) SYNTHESIS OF MODEL COMPOUND, *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Pro-$CONH_2$ 119.

Before we started to synthesize target hexapeptide **1**, *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Pro-$CONH_2$ **119** had been synthesized as a model (scheme 59). Fmoc protecting group was removed from Rink amide resin with two treatments of 20% piperidine in DMF to obtain free *N*-terminus of solid support. Fmoc-*L*-amino acids were activated with HOBt and DIC in the presence of DIPEA in DMF except Fmoc-*L*-Arg(Pbf)-OH which was activated by HBTU in the presence of DIPEA in DMF. The Fmoc-deprotecting and coupling reaction were repeated. The beads were then subjected to stepwise assembly of Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH, respectively. The coupling and deprotecting reaction were monitored by Kaiser test for primary amine residue and choranil test for secondary amine residue. *N*-terminal of resin-tripeptide was capped with 50% Ac_2O in pyridine. The peptide residue was cleaved from solid support and Pbf protecting group was removed at

the same time with TFA/H_2O/TIS mixture. The crude residue was precipitated to obtain compound **119** as a white solid in high yield.

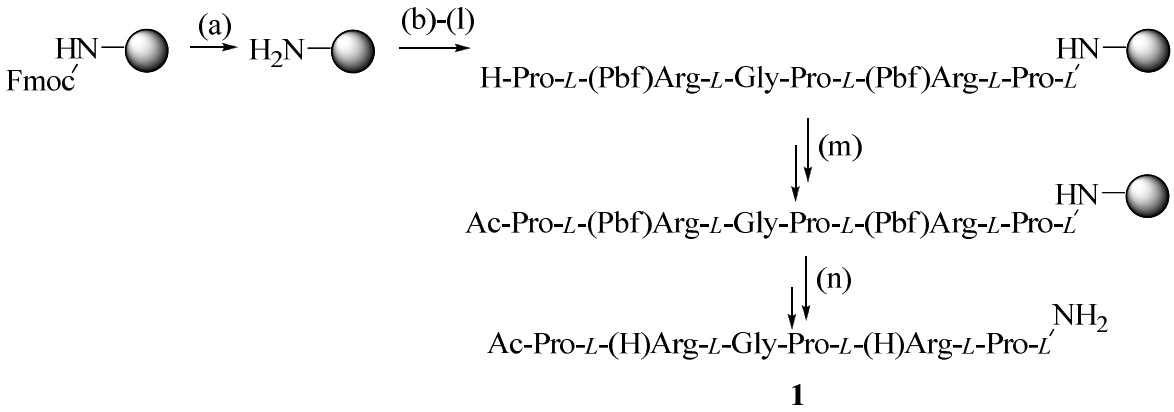


a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) 50% Ac_2O/pyridine (3.0 equiv), i) TFA: TIS: H_2O (96:2:2), 84%.

Scheme 59: Synthesis of *N*-Ac-L-Pro-L-Arg(H)-L-Pro-$CONH_2$ **52**.

3.3) SYNTHESIS OF TARGET HEXAPEPTIDE, *N*-Ac-L-Pro-L-Arg(H)-Gly-L-Pro-L-Arg(H)-L-Pro-NH_2 1.

After successfully synthesizing *N*-Ac-L-Pro-L-Arg(H)-L-Pro-$CONH_2$ **119**, we proceeded with the synthesis of our target hexapeptide, *N*-Ac-L-Pro-L-Arg(H)-Gly-L-Pro-L-Arg(H)-L-Pro-$CONH_2$, **1** using analogous synthetic procedure (scheme 60). The beads were then subjected to stepwise assembly of Fmoc-L-Pro-OH, Fmoc-L-Arg(Pbf)-OH and Fmoc-L-Pro-OH, Fmoc-Gly-OH, Fmoc-L-Arg(Pbf)-OH and Fmoc-L-Pro-OH, respectively. The coupling and deprotecting reaction were monitored by Kaiser test for primary amine residue and choranil test for secondary amine residue. *N*-terminal of resin-tripeptide was capped with 50% Ac_2O in pyridine. The peptide residue was cleaved from solid support and Pbf protecting group was removed at the same time with TFA/H_2O/TIS mixture. The crude residue was purified by reverse phase flash column chromatography and precipitated to obtain compound **1** as a white solid in high yield.



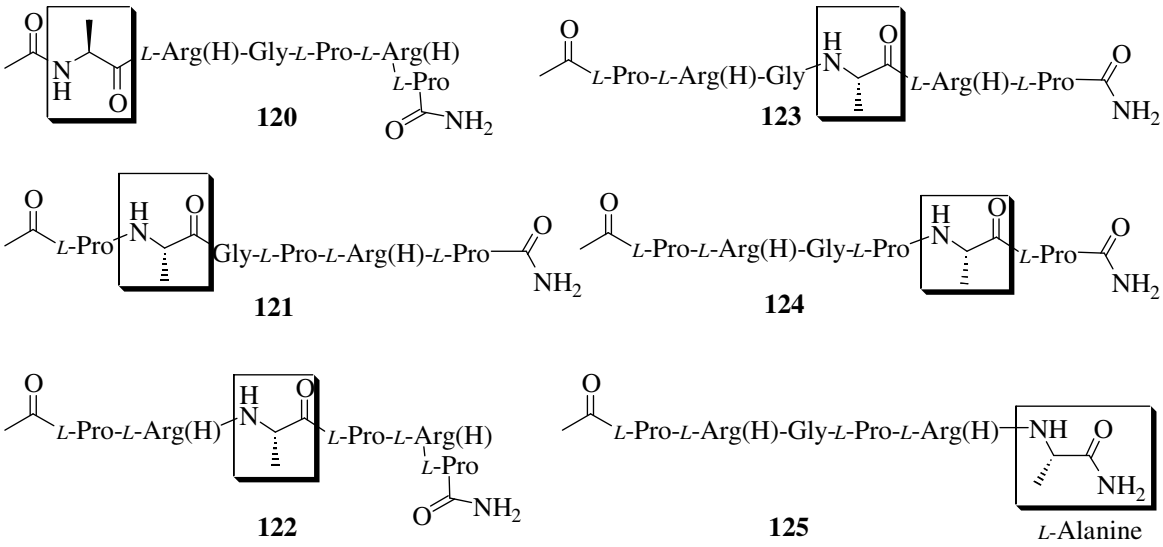
1

a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-Gly-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2), 32%.

Scheme 60: Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-$CONH_2$ **1**.

3.4) SOLID PHASE PEPTIDE SYNTHESIS OF *L*-ALANINE SCANNING COMPOUNDS 120-125.

According to the previous section, target hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-$CONH_2$**, 1** was successfully synthesized by solid phase synthesis. We found that compound **1** was a lead peptide which obtained anticancer activity without killing normal cells. "Alanine scan" analysis on the peptide **1** was performed by sequentially replacing each amino acid with *L*-alanine clearly demonstrated that residues Pro-1, Arg-2, Pro-3, Gly-4, Arg-5 and Pro-6 were critical for cell binding. The peptides **120**-**125** were synthesized by an analogous methodology using stepwise *C*-terminus elongation as described in section 3.3. All synthetic schemes of *L*-alanine scanning compounds **120**-**125** are shown in appendix 2 and the full chemical structures are shown in scheme 61.



Scheme 61: The chemical structures of *L*-alanine scanning compound **120-125.**

The hexapeptides **120**-**125** were prepared through solid phase synthesis starting from Rink amide resin. Fmoc-protected amino acids were condensed using DIC/HOBt or HBTU/HOBt in the presence of DIPEA and DMF. 20% Piperidine in DMF was used to remove the Fmoc-protecting group. The coupling and deprotecting reaction were monitored by Kaiser test for primary amine residue and choranil test for secondary amine residue. *N*-terminal of hexapeptide chain was capped with 50% Ac_2O in pyridine and peptide was cleaved from solid support and Pbf protecting group was removed same time with TFA/H_2O/TIS mixture. All final products were purified by reverse phase flash column chromatography.

In conclusion, target hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe **1** and its alanine scanning analogues **120-125** were successfully synthesized by solid phase peptide method for biological assay. The details of biological assays are explained in chapter 8. The structural characterization was then achieved by NMR (^{1}H and ^{13}C) spectra to confirm structure and LRMS and HRMS was done to confirm mass of the peptides. Especially, HPLC was done to confirm purity of the compound (appendix 4).

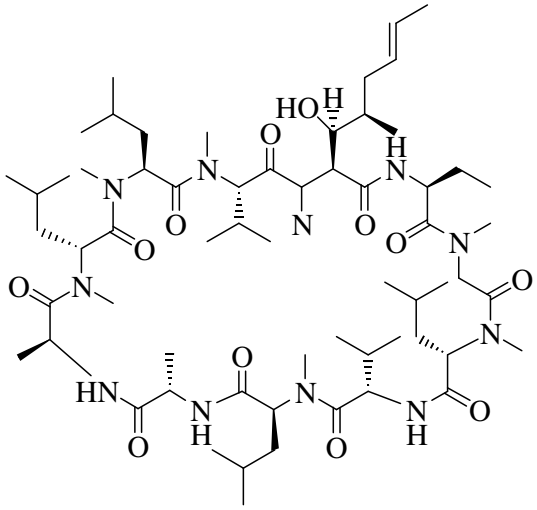
CHAPTER 4: CYCLIC PEPTIDES MACROCYCLIZATION

4.1) INTRODUCTION

Naturally occurring cyclic peptides that have been isolated from a variety of sources including bacteria, marine organisms and plants [88] exhibit a wide range of biological property. Synthetic cyclic peptides have several advantages as drug candidates [89]. The macrocyclization of peptides are synthesized to constrain its active conformation and increased its potency and also specificity. In addition, these cyclic compounds do not have any charges at *N*- and *C*-terminal and lack of zwitterionic character; therefore, they are more hydrophobic and membrane permeable. There are three general types for peptide cyclizations such as the backbone head-to-tail cyclization, side chain-to-side chain ring closure (disulfide or amide bond formation) and head-to-side chain or side chain-to-tail cyclization. Recently, peptide macrocyclization reactions have been extensively reviewed [90]. Especially, the methods for the backbone cyclization of peptides have been reviewed by Lambert *et al.* [91]. However, the head-to-tail cyclization of a linear peptide is occurred in low yield when compared to intermolecular bond formations. A lot of side reactions, such as dimerization and cyclodimerization of linear peptide precursors are observed for certain ring sizes [92]. These problems can be solved by performing the cyclization reaction under high dilution conditions in 10^{-3} to 10^{-4} M solution and slow addition of both coupling reagent and peptide precursor by syringe pump [93].

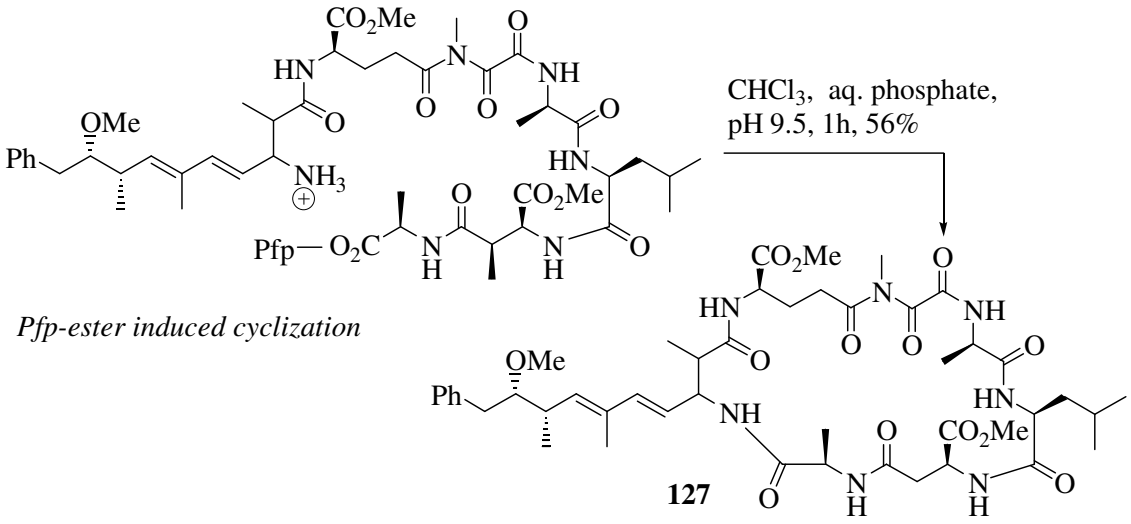
4.1.1) Literature reviews of cyclic peptides synthesis.

Cyclosporines, for example, are family of cyclic undecapeptides which display variety of active biological activities [94]. Rich, D. H. *et al.* synthesized D-Lys^8-cyclosporine analogue **126** by the coupling reaction between D-Lys and L-Ala *via* $(PrPO_2)_3$/DMAP activation in dilute solution to achieve cyclic peptide 68% yield (scheme 62).



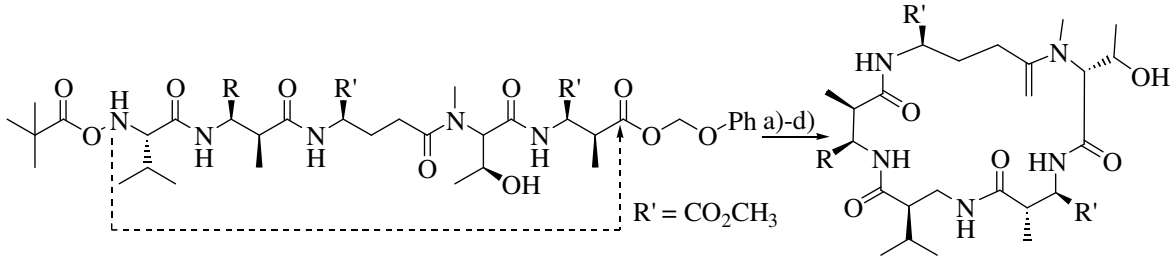
Scheme 62: *D*-Lys[8]-cyclosporine analogue **126** was cyclised by $(PrPO_2)_3$/DMAP activation in dilute solution**.**

A pentafluoroester method often gives better results than other active ester methods and has been applied to many cyclopeptide syntheses [95]. Chamberlin, A. R. *et al.* [96] successfully developed the cyclization method using pentafluorophenol (Pfp-OH) to activate microcystin LA precursor and followed by rapid ring closure in two-phase $CHCl_3$ at pH 9.5 of phosphate buffer system. The cyclic compound **127** was obtained in 56% yield within 1 h (scheme 63).



Scheme 63: Pfp-ester induced cyclization of microcystin LA **127**.

The Pfp-ester method was also chosen by Schreiber, S. L. *et al.* [97] for their motuporin ring closure **128** between Val and methyl-*D*-Asp. Cyclization required several days at high dilution to obtain cyclic product **128** in 55% (scheme 64).



Muturin **128**

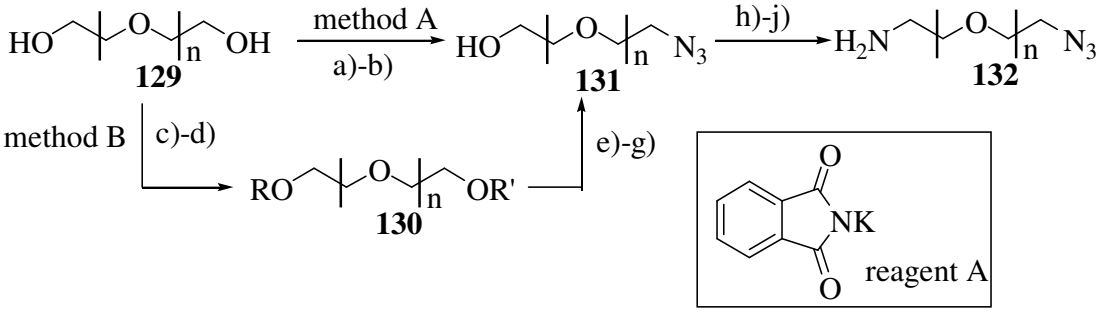
a) Zn, HOAc, b) Pfp-OH, DCC, c) TFA, CH_2Cl_2, d) DIPEA, DMAP, 55% for 4 steps.

Scheme 64: Pfp-ester induced cyclization of motuporin **128**.

4.1.2) Literature reviews of modified polyethylene glycol (PEG) synthesis.

A new strategy for modification of peptides by polyethylene glycol (PEG) is developed. The PEG derivatives are ideal for the bifunctional molecules which can be used in antibody production [98], drug delivery [99], protein immobilization [100] and enzyme study [101]. The PEG derivatives can be easily coupled with peptide by amide bond formation and have been used as a biocompatible water soluble, flexible, variety of lengths, inexpensive and chemically inert spacers because the space length can be readily varied by selection of the appropriate commercial precursor [102].

Bednarski, M. D. *et al.* [103] synthesized heterobifunctional PEG derivative **132** which was prepared from commercially available PEG **129.** Azido alcohol intermediate **131** could be synthesized by two methods. The first method involved mesylation by MsCl and followed by azide formation. Secondly, PEG **129** was monosilylated of by TBDMSCl, followed by mesylation with MsCl and deprotected with n-Bu_4NF to provide compound **131**. Azido alcohol **131** was then mesylated by MsCl and followed by Grabrial amine synthesis to obtain compound **132** which contained a free amine and could be directly conjugated to molecules by an amide linkage (scheme 65).

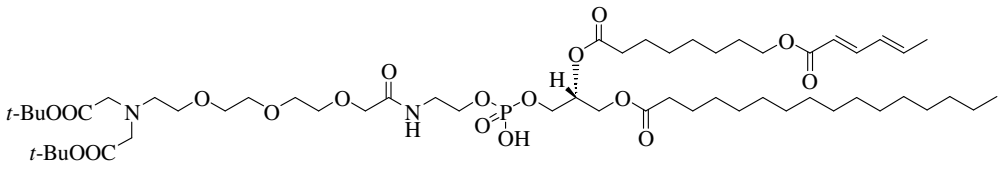


Method A: *a) MeCl, Et_3N, b) NaN_3, EtOH*

Method B: *c) NaH, THF, d) TBDMSCl, e) MsCl, Et_3N, f) NaN_3, EtOH, g) n-Bu_4NF, THF, h) Mscl, Et_3N, i) reagent A, DME, j) NH_2NH_2.*

Scheme 65: The synthesis of heterobifunctional PEG derivative **132**.

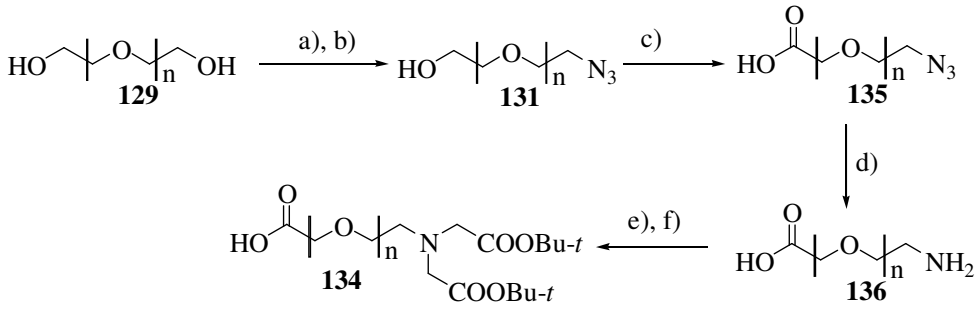
O'Brien, D. F. *et al.* [104] synthesized metal ion chelating lipids **133** (scheme 66) which could be used for the formation of functionalized lipid surfaces. This lipid was based on water soluble, flexible and inert spacer precursor **134** which could be synthesized from commercially available PEG **129**.



Lipid 133

Scheme 66: Chemical structure of metal ion chelating lipid **133.**

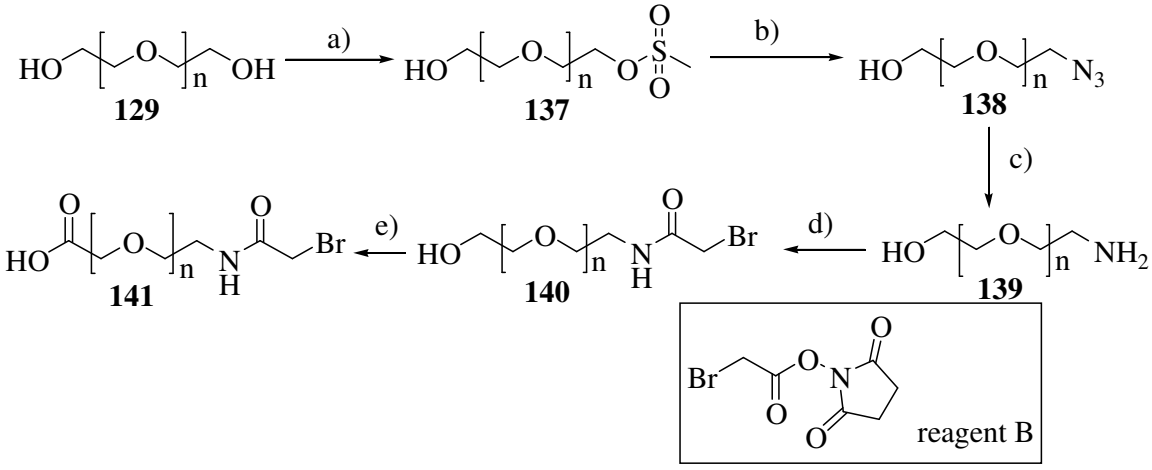
The commercially available of PEG **129** was monomesylated with MsCl and followed by azide formation with NaN_3 under reflux condition to obtaine azide alcohol **131** in reasonable yield (75%). This intermediate **131** was oxidized by $BrCH_2COOH$ at room temperature and followed by azide reduction which could be accomplished by mild, biocompatible reagent such as 1,3-propanedithiol to provide heterobifunctional amino acid **136**. Precursor **134** was prepared from alkylation of compound **136** with $BrCH_2CO_2{}^tBu$ in DMF in the presence of K_2CO_3 and KI and followed by hydrolysis with 1 N NaOH in EtOH (scheme 67).



a) MsCl, Et_3N, THF, b) NaN_3, EtOH, reflux, c) $BrCH_2COOH$, KOH, DMF, d) $HS(CH_2)_3SH$, Et_3N, MeOH, e) $BrCH_2CO_2{}^tBu$, K_2CO_3, KI, DMF, f) 1N NaOH, EtOH.

Scheme 67: The synthesis of heterobifunctional PEG **134**.

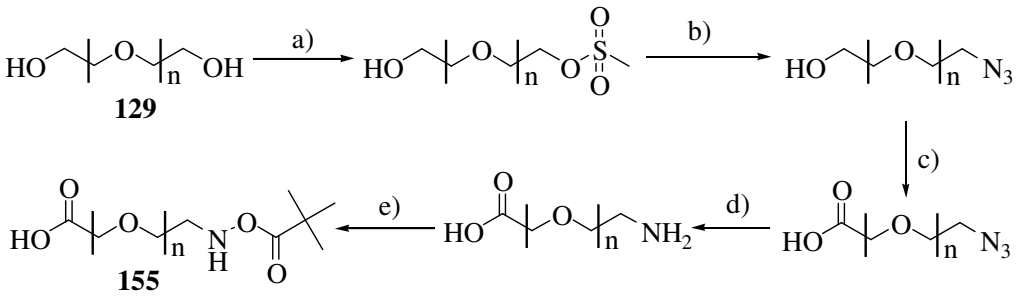
Sulfonate ester had been extensively used as a good leaving group for substitution reactions at both of terminal ends of ethylene glycol. Especially, mesylate had often been used as starting material for the preparation of a variety of functionalized oligo- and poly (ethylene glycol). Schuber, F. *et al.* [105] synthesized a hydrophobic and flexible heterobifunctional reagent **141**. An amino alcohol **139** was prepared by monomesylation of commercially available PEG **129** with methansulfonylchloride at room temperature overnight, followed by azide formation with sodium azide under reflux condition to obtain mono-azide PEG **138** which was reduced by PPh_3 in the presence of water to obtain compound **139**. A heterobifunctional compound **141** was prepared by the reaction between amino alcohol PEG **139** and *N*-hydroxysuccinimidyl ester of bromoacetate at room temperature to obtain intermediate **140** which oxidized the alcohol functional group by Jones reagent (scheme 68).



a) Methanesulfonyl chloride, NEt_3, THF, RT, overnight, b) NaN_3, CH_3CN, reflux, 36 h., PPh_3, H_2O, THF, RT, 10 h., d) reagent B, $CHCl_3$, overnight, RT, e) Jones reagent, acetone, 1 h., RT.

Scheme 68: Synthesis of heterobifunctional PEG **141**.

From previous study in our group, synthetic method to synthesize a modified PEG **155** and its derivatives (n = 1, 2, 3 and 4) was developed by Dr. S. Dixon. Commercially available polyethylene glycol **129** was chosen to use as a starting material and was easily to functionalize both *N*- and *C*-terminus of molecule (scheme 69). The monomesylation of commercially available PEG **129** was done by methanesulfonyl chloride at room temperature overnight and a subsequent reaction by sodium azide under reflux condition to obtain azide alcohol which was oxidised with Jones reagent to obtain carboxylic acid. Azide reduction with 1,3-propanedithiol and *N*-Boc protection of the resulting primary amino group were obtain modified PEG **155** in reasonable yield. In our project, the disubstituted PEG **155** (n = 3) was synthesized by using an analogous synthetic method. The results and discussion were described in section 4.2.2.



a) Methanesulfonyl chloride, NEt_3, THF, RT, overnight, 21%, b) NaN_3, CH_3CN, reflux, 36 h., 88%, c) Jones reagent, acetone, 1 h., RT, 63%, d) 1,3-propanedithiol, NEt_3, MeOH, RT, 3 days, e) di-tert-carbonylcarbonate, KOH, H_2O, THF, RT, overnight, 81% over two steps.

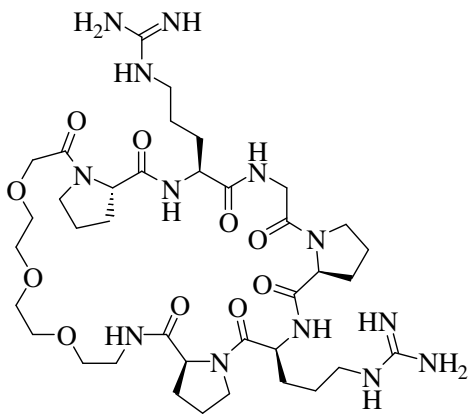
Scheme 69: Synthesis of modified PEG **155**.

4.2) RESULTS AND DISCUSSION

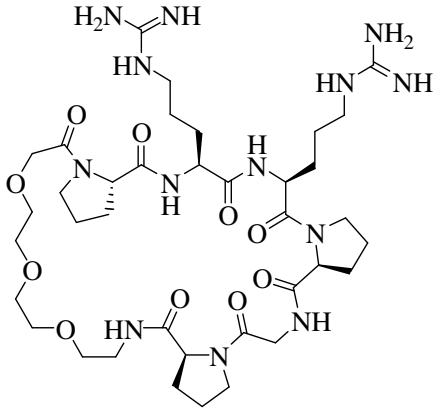
History: from previous study in our group, a computational model study of CDK4 was investigated by Dr. J. Essex. As result found that the length from *N*- to *C*-terminus of peptide chain in stable conformation was 17.5 Å which was similar with the length of

polyethylene glycol (n = 3). Therefore, tetraethylene glycol is chosen to use in this work as a tether. Dr. S. Dixon developed the synthetic method to modify both ends of polyethylene glycol and also varied number of ethylene part from n = 1, 2, 3 and 4. The procedure of cyclic peptide synthesis was also developed by Dr. S. Dixon as well by coupling both ends of modified PEG (n = 1, 2, 3 and 4) with linear peptide **64**. A series of cyclic peptides [*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro] were successfully synthesized under high dilution conditions and slow addition method. The cytotoxic activities of these compounds were studied by clonogenic assay against RT112 bladder cancer cells. As results found that cyclic peptide **161** (n = 3) showed an excellent anticancer activity in 12 days.

In this work, the cyclic peptide **161** (n = 3) was repeated to synthesize using an analogue synthetic method. The linear peptide **64**, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, was used as a precursor of cyclic peptide synthesis and coupled with modified PEG tether **155** to obtain the cyclic peptide **161** in reasonable yield. Cytotoxic activity of the cyclic peptide **161** was studied by clonogenic assay against both of RT112 bladder cancer and fibroblast cells. In parallel, a cyclic peptide **168** was synthesized using an analogue synthetic method. A linear hexapeptide precursor **150**, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe, that was achieved by solution phase method, coupled with modified PEG tether **155** to obtain cyclic peptide **168** in reasonable yield. From the previous study by H. Warenius, the peptide sequence *L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro did not show any biological activity with cancer cell lines. That's why the cyclic peptide **168** was synthesized and used as a negative controlled peptide against both of RT112 bladder cancer and fibroblast cells in biological assay. The details of biological assays of both cyclic compounds **161** and **168** are described in chapter 8. The chemical structures of these compounds are shown in scheme 70.



161

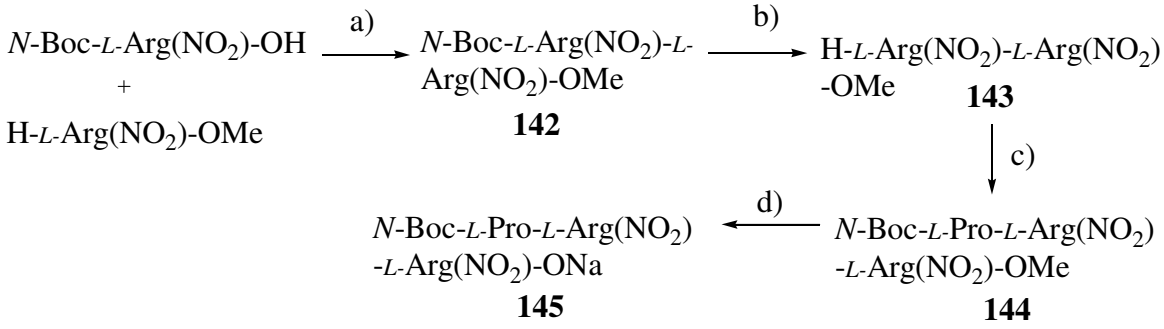


168

Scheme 70: Chemical structures of cyclic peptides **161** and **168**.

4.2.1) Synthesis of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2-*L*-Pro-Gly- *L*-Pro-OMe 150.

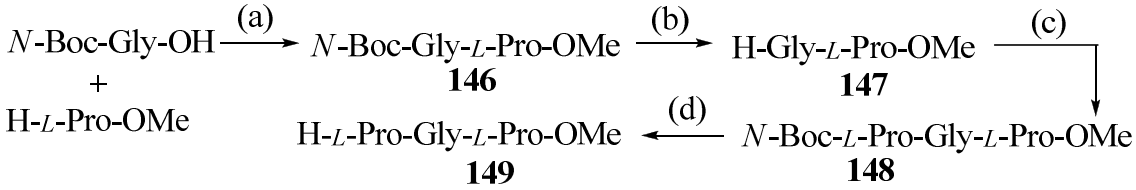
Hexapeptides **150** was synthesized by conventional solution phase methods using a fragment condensation strategy and could be used as major core for macrocyclization. Tripeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-ONa, **145** was synthesized according to the steps shown in scheme 71. Dipeptide **142** was obtained as a white solid in high yield from the coupling reaction between the commercially available *N*-Boc-*L*-Arg(NO_2)-OH and H-*L*-Arg(NO_2)-OMe. The *N*-Boc deprotection of dipeptide **142** was done with 4M HCl in dioxane, to provide free *N*-terminus compound **143** as a white solid in high yield and could be used in the next step without further purification. The tripeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-OMe, **144** was prepared under the same coupling conditions between commercially available *N*-Boc-*L*-Pro-OH and dipeptide **143** to give compound **144** as a white solid in moderate yield. Hydrolysis reaction was followed to remove *C*-terminus protecting group (OMe) using 1.0 equiv of 1M aq. NaOH in ethanol, to provide tripeptide precursor **145** as a white solid in medium yield and was used in the next step without further purification.



a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, 70%, b) 4M HCl in dioxane (3.0 equiv), 92%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, 52%, d) 1M aq. NaOH in EtOH (1.0 equiv), 87%.

Scheme 71: Synthetic of tripeptide precursor, *N*-Boc-L-Pro-L-Arg(NO_2)-L-Arg(NO_2)-ONa, **145**.

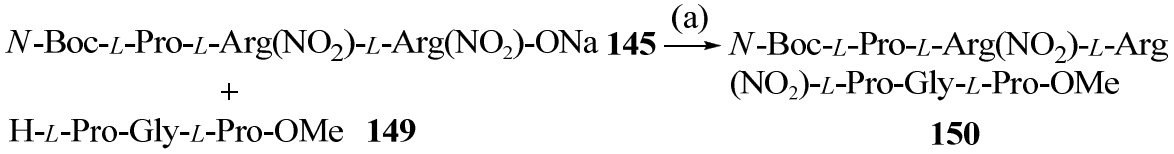
Tripeptide, H-L-Pro-L-Gly-L-Pro-OMe, **149** was synthesized according to the steps shown in scheme 72. Dipeptide **146** was obtained as colourless oil in resonable yield from the general coupling reaction under the same conditions between the commercially available *N*-Boc-Gly-OH and H-L-Pro-OMe. The *N*-Boc deprotection of dipeptide **146** was done with 4M HCl in dioxane, to provide free *N*-terminus compound **147** as colourless oil in high yield. The tripeptide, *N*-Boc-L-Pro-Gly-L-Pro-OMe, **148** was prepared under the same coupling conditions between commercially available *N*-Boc-L-Pro-OH and dipeptide **147**. Because there were a lot of products in crude residue and then after purified by column chromatography, *N*-Boc-L-Pro-Gly-L-Pro-OMe **148** was obtained as colorless oil in low yield. The *N*-Boc deprotection of tripeptide **148** was done with 4M HCl in dioxane, to provide free *N*-terminus compound **149** as colourless oil in high yield. All products in this scheme were UV inactive compounds then we used ninhydrin stain for TLC monitoring.



a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, 60%, b) 4M HCl in dioxane (3.0 equiv), 95%, c) N-Boc-L-Pro-OH (1.0 equiv), HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (4.8 equiv), DMF, RT, 43%, d) 4M HCl in dioxane (3.0 equiv), 90%.

Scheme 72: Synthesis of tripeptide precursor, H-L-Pro-Gly-L-Pro-OMe **149**.

Finally, hexapeptide, *N*-Boc-L-Pro-L-Arg(NO_2)-L-Arg(NO_2-L-Pro-Gly-L-Pro-OMe, **150** was synthesized according to the steps shown in scheme 73 by the coupling under the same condition between the tripeptide precursors **145** and **149** to give compound **150** as a white solid in medium yield.

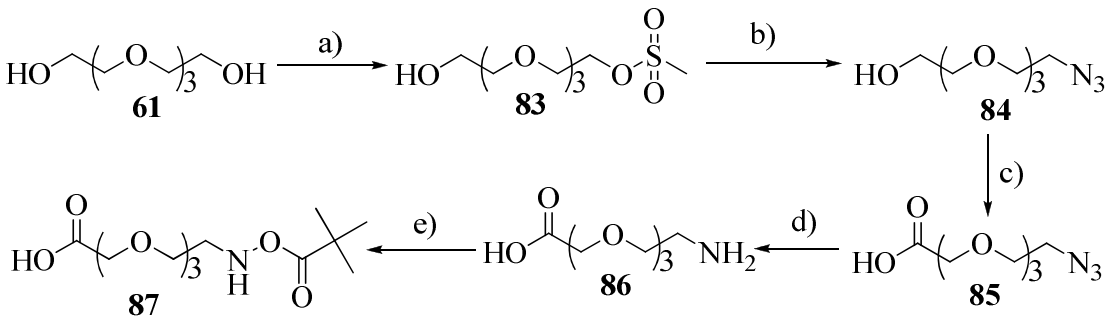


a) HOBt (1.2 equiv), DCC (1.0 equiv), DIPEA (10.0 equiv), DMF, RT, 51%.

Scheme 73: Synthesis of hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe, **150**.

4.2.2) Synthesis of poly(ethyleneglycol) (PEG) tether 155.

The PEG tether **155** was synthesized according to the steps shown in scheme 74. The commercial available tetraethylene glycol **129** was converted into good leaving group with methanesulphonyl chloride to obtain monomesylate derivative **151** in low yield, which was converted into the 2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy] ethanol **152** in high yield by reaction with sodium azide in CH_3CN under reflux for 36 h. Azide **152** was conveniently oxidized into carboxylic acid **153** in medium yield with Jones's reagent which freshly prepare by a mixture of chromic trioxide in diluted sulfuric acid.

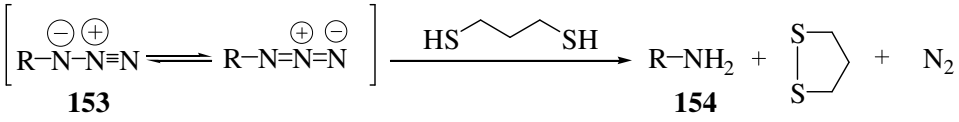


a) Methanesulfonyl chloride, NEt_3, THF, RT, overnight, 21%, b) NaN_3, CH_3CN, reflux, 36 h., 88%, c) Jones reagent, acetone, 1 h., RT, 63%, d) 1,3-propanedithiol, NEt_3, MeOH, RT, 3 days, e) di-tert-carbonylcarbonate, KOH, H_2O, THF, RT, overnight, 81% over two steps.

Scheme 74: The synthesis of disubstituted PEG **155.**

Compound **154** was prepared by reduction with 1,3-propanedithiol [106]. Reaction mixture was stirred at room temperature for 3 days. The mixture was diluted with water and extracted with diethyl ether. The aqueous layer was evaporated *in vacuo* to obtain

compound **154** as colorless oil in high yield and crude residue was used in the next step without further purification. The facile reduction by 1,3-propanedithiol was reported as the use of mild, biocompatible reagent which could be reduced azides at room temperature. General chemical equation of azide reduction is shown in scheme 75 [107].



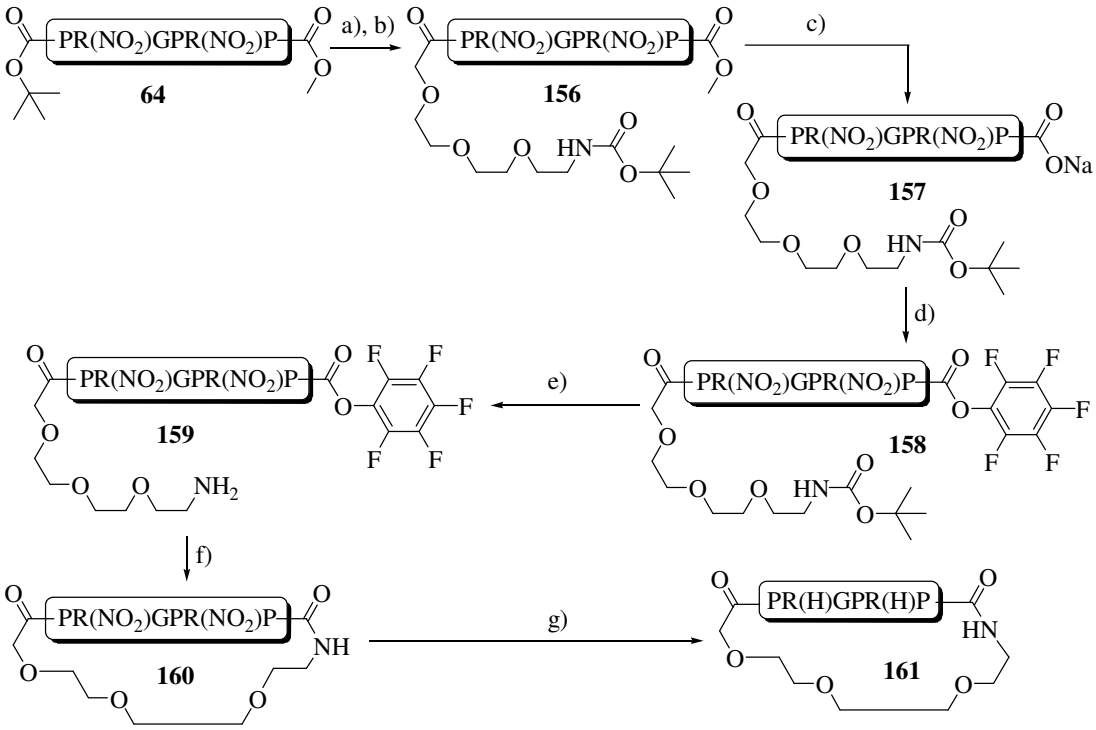
Scheme 75: Azide reduction by 1,3-propanedithiol.

Finally, 2,2-Dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-oic acid **155** was prepared from hetero-bifunctional compound **154** by *N*-Boc protection chemistry with di-*tert*-butyl dicarbonate and KOH (aqueous preparation) in THF. Crude residue was purified and obtained the modified polyethylene glycol **155** as colorless oil in high yield.

4.2.3 Synthesis of cyclic peptides

Cyclic peptide **161** was synthesized according to the steps shown in scheme 76. *N*-Boc deprotection of peptide **64** was done by 4 M HCl in dioxane to obtain free *N*-terminus peptide as a white solid in high yield. The desired product could be used in the next step without further purification. Consequently, coupling reaction between activated heterobifunctional amino acid **155** by HOBt and DCC and free *N*-terminus peptide was done under general coupling conditions. The crude residue was then purified by column chromatography to obtain compound **156** in medium % yield. Hydrolysis of methyl ester peptide **156** was readily achieved upon treatment with 1M NaOH (1.5 equiv, aqueous preparation) in water for 3 h. All solvents were removed *in vacuo* and crude residue was precipitated with CH_2Cl_2, MeOH and diethyl ether to obtain compound **157** as a white solid in high yield. Activated ester **158** was synthesized by using Pfp-OH, DMAP and EDC. Reaction mixture was stirred at room temperature for 12 h. All solvents were removed *in vacuo* to provide crude residue **158** as pale yellow oil in medium yield. To prevent the removal of Pfp group, the crude residue **158** was used in the next step without further precipitation. *N*-Boc deprotection of the crude pfp ester **158** was done with 4 M HCl in dioxane at room temperature. All solvents were removed *in vacuo* to obtain crude product **159** as pale yellow oil and could be used in the next step without further precipitation. With

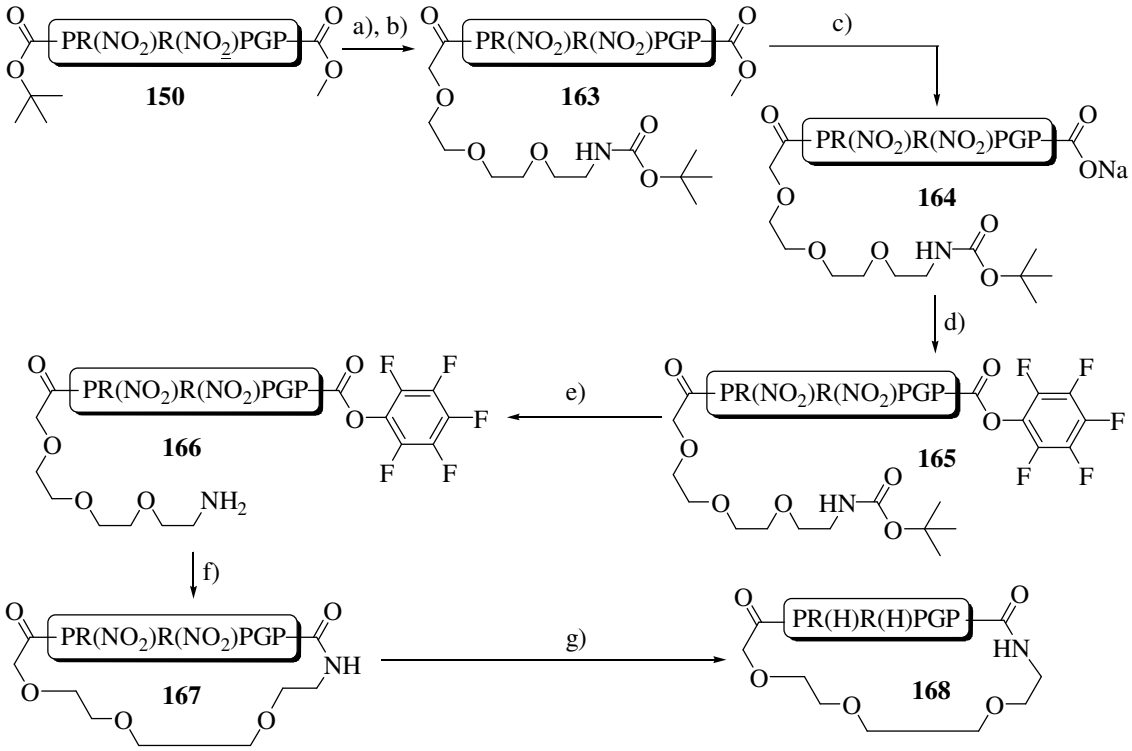
a ready supply of intermediate **159** in hand, macrocyclization was taken under slow addition by syringe pump (6 h) and high dilution (0.01 M in CH_3CN) in reflux conditions. The crude residue was purified by column chromatography and precipitated with acetonitrile and diethyl ether to obtain product **160** as a white solid in low yield. The purity of product **160** was confirmed by a single chromatogram of LCMS (ES^+). Finally, protecting groups of guanidine side chain (-NO_2) were removed by catalytic hydrogenolysis of compound **160** with 10% Pd/C, H_2 in the mixture of MeOH: AcOH (3:1 ratio). Reaction mixture was stirred at room temperature for 2 days and monitored by LRMS until there was no starting material left. All catalyst was removed by suction filtration through a celite pad, washed with MeOH and triturated with diethyl ether to provide final product **161** as a white solid in high yield. The single chromatogram of LCMS (ES^+) confirmed purity of cyclic peptide.



a) 4M HCl/dioxane (3.0 equiv), b) 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-oic acid (1.0 equiv), HOBt (1.2 equiv), DCC 1.0 equiv), DIPEA (10.0 equiv), DMF, 40%, c) 1M aq. NaOH (1.5 equiv), H_2O, 95%, d) pentafluorophenol (2.0 equiv), DMAP (0.01 equiv), EDC (2.0 equiv), DMF, 93%, e) 4M HCl in dioxane, 98%, f) DIPEA, MeCN, 10%, g) 10% Pd/C, H_2, AcOH: MeOH (1: 3), 72%.

Scheme 76: Synthesis of cyclic peptide **161**.

After we successfully synthesized cyclic peptide **161**, we proceeded with the synthesis of the cyclic peptide **168** using an analogous synthetic procedure (scheme 77). The purity of final compound **168** was confirmed by single chromatogram of LCMS (ES^+).



a) 4M HCl/dioxane (3.0 equiv), b) 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-oic acid (1.0 equiv), HOBt (1.2 equiv), DCC 1.0 equiv), DIPEA (10.0 equiv), DMF, 40%, c) 1M aq. NaOH (1.5 equiv), H_2O, 95%, d) pentafluorophenol (2.0 equiv), DMAP (0.01 equiv), EDC (2.0 equiv), DMF, 93%, e) 4M HCl in dioxane, 98%, f) DIPEA, MeCN, 10%, g) 10% Pd/C, H_2, AcOH: MeOH (1: 3), 72%.

Scheme 77: Synthesis of cyclic peptide **168**.

The cyclic peptide samples **161** and **168** were dissolved in both of DMSO-d^6 or MeOH-d^4. ^{1}H NMR spectra of the peptides were recorded at 400 K. Unfortunately we were unable to assign the proton peaks because the quality of ^{1}H NMR spectra was not good enough, although we increased number of running scan up to 1K. We also could not be increased quantity of cyclic peptides to improve the quality of NMR data because we needed to save both of these cyclic peptides **161** and **168** for the biological assays against RT112 bladder cancer and MRC5-hTERT fibroblast cells. As results, we realized that there were no NMR

(^{1}H and ^{13}C) spectra to confirm the structure of both of final compounds **161** and **168**. Then, the most characterization of this synthetic method was carefully done by LRMS (ES^{+}) step by step and LCMS to confirm the purity of compounds (appendix 5, 6 and 7).

In conclusion, we successfully synthesized a linear hexapeptide precursor, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe, **150** by solution phase method and modify PEG **155** by the method which was previously developed in our laboratory. The anticancer activity of these cyclic peptides **161** and **168** were studied by clonogenic assay. The details are discussed in chapter 8.

CHAPTER 5: EXPERIMENT

5.1 GENERAL TECHNIQUES

All of the commercially available amino acids in this work were *L*-configuration. Thin layer chromatography was conducted on silica pre-coated plates (Merck silica gel 60 F254, aluminium backed) and the spots visualised with UV light (254nm) or ninhydrin. Low resolution mass spectra were recorded on a Waters ZMD mass spectrometer, single quadrupole, 2700 autosampler. High resolution mass spectra were recorded on VG analytical 70-250-SE double focusing mass spectrometer. Values of *m/z* were reported in atomic mass units and the peak intensity relative to the base peak was reported in parenthesis. High pressure liquid chromatography (HPLC) was recorded on a C-18 column (C18, 5-10 μ, column dimension (9.4 mm × 25 cm) using 0.1% TFA/H_2O and 0.1% TFA/CH_3CN gradients (0-100%) and using detection at 226 nm. The IR spectra were recorded on a ThermoNicolet 380 FT-IR spectrometer with a Smart Orbit Goldengate attachment. Absorption was given in wavenumbers (cm^{-1}) and the abbreviations used to identify peak intensities: s = strong, m = medium, w = weak and br = broad. UV spectra were recorded with a Shimadzu UV-1601 spectrometer in quartz cells. NMR spectra were recorded on a Bruker AV 300 (^1H, 300 MHz; ^{13}C, 75 MHz) and on a Bruker DPX 400 (^1H, 400 MHz; ^{13}C, 100 MHz). Chemical shifts (δ) were given in parts per million relative to the peak for the residual solvent. NMR peak positions were recorded relative to the residual dimethylsulfoxide (d^6-DMSO), chloroform ($CDCl_3$) and methanol (d^4-MeOD) signal, using the following abbreviations: singlet (s), doublet (d), double of doublet (dd), triplet (t), quartet (q), pentet (p), double of triplet (dt), multiplet (m), broad singlet (br). ^{13}C peaks were indicated using the following abbreviations: quarternary carbon (C), tertiary (CH), secondary (CH_2) and primary (CH_3). Uncorrected melting points were determined with open capillary tubes using a Gallenkamp Electrothermal Melting Point Apparatus.

5.2 GENERAL PROCEDURE FOR SOLUTION PHASE PEPTIDE SYNTHESIS

Peptides were synthesized by conventional solution phase methods using a fragment condensation strategy. A *tert*-butyloxycarbonyl group (Boc) was used for *N*-terminal protection and *C*-terminal was protected as a methyl ester (OMe). Deprotection at the *N*-terminus was performed using 20% TFA in CH_2Cl_2 or 4M HCl in dioxane (3.0 equiv) and

saponification by 1 M NaOH was done to remove the *C*-terminal protecting groups. Couplings were activated by DCC and HOBt in the presence of DIPEA in DMF. All *N*-Boc-protected-*L*-amino acids were used from NovaBiochem without further purification, except *N*-Boc-*L*-Arg(NO_2)-OH was used from Aldrich and recrystallized from EtOAc before used. Nitro group (-NO_2) was used as protecting group of guanidine side chain of arginine (Arg). DCC, HOBt and DIPEA were purchased from Aldrich, Molekula and Fluka, respectively and used without further purification.

5.2.1) Coupling reaction by DCC and HOBt.

To a stirred solution of carboxy component (1.0 mmol) in DMF (1 mL of DMF per 0.5 g of amino acid or peptide) was slowly added HOBt (1.2 equiv, 1.2 mmol) and DCC (1.0 equiv, 1.0 mmol), respectively. Amino component (1.0 mmol) was dissolved in DMF (1 mL of DMF per 0.5 g of amino acid or peptide) in another portion and added DIPEA (4.8 equiv, 4.8 mmol) to generate free amine residue. This amino solution was added dropwise at 0 °C to a stirred carboxy solution. The reaction mixture was warmed to room temperature and stirred overnight. White precipitate (DCU) was removed by suction filtration and washed with DMF (10 mL required for 1.0 mmol preparation). All solvents were removed under reduced pressure. The crude residue was dissolved with CH_2Cl_2 and washed with small amount of cold water (5 mL, required for 1.0 mmol preparation), 10% cold $NaHCO_3$ (5 mL, required for 1.0 mmol preparation), 10% cold citric acid (5 mL, required for 1.0 mmol preparation), dried with anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude residue was purified by column chromatography on silica gel and precipitated by CH_2Cl_2, MeOH and Et_2O.

Note: 10.0 equiv of DIPEA was used for hexapeptide coupling and crude residue was purified by column chromatography without washing with CH_2Cl_2, water, 10% cold and 10% cold citric acid.

5.2.2) *N*-Boc deprotection by 20% TFA in CH_2Cl_2.

The *N*-Boc-protected peptide (1.0 mmol) was treated with 20% trifluoroacetic acid (TFA) in dichloromethane (CH_2Cl_2) (3.0 equiv, 3.0 mmol) at 0 ºC. The solution was stirred at

room temperature overnight. All solvents were removed by toluene azeotrope under vacuum and desired product was precipitated from CH_2Cl_2, MeOH and Et_2O.

5.2.3) *N*-Boc deprotection by 4M HCl in dioxane.

The *N*-Boc-protected peptide (1.0 mmol) was treated with 4 M HCl in dioxane (3.0 equiv, 3.0 mmol) at 0 ºC. The solution was stirred at room temperature for 2 h. All solvents were removed under vacuum and desired product was precipitated from CH_2Cl_2, MeOH and Et_2O.

5.2.4) Saponification of peptide ester.

To a stirred solution of ester peptide (1.0 mmol) in ethanol (2 mL, required for 1.0 mmol preparation) was added 1 M NaOH (1.0 equiv, aqueous preparation, 1.0 mmol) at room temperature. The reaction mixture was stirred overnight. All solvents were removed *in vacuo* and desired product was precipitated from CH_2Cl_2, MeOH and Et_2O.

5.2.5) *N*-Acetylation of peptide.

To a stirred solution peptide (1.0 mmol) in DMF (5 mL required for 1.0 mmol preparation) was added triethylamine (3.0 equiv, 3.0 mmol), 4-dimethylaminopyridine (DMAP) (0.05 equiv, 0.05 mmol), and then acetic anhydride (3.0 equiv, 3.0 mmol) at 0 ^{o}C. The reaction mixture was warmed to room temperature and stirred overnight. All solvents were removed under reduced pressure. The crude residue was washed with CH_2Cl_2 (3 x 10 mL, required for 1.0 mmol preparation) and cold H_2O (10 mL, required for 1.0 mmol preparation), and purified by column chromatography. Desired product was precipitated from CH_2Cl_2, MeOH and Et_2O.

5.2.6) Amide formation of peptides.

Peptide (1.0 mmol) was dissolved in 35% NH_4OH (5 mL, required for 1.0 mmol preparation) and stirred at 0 °C for 1 h. The reaction mixture was warmed to room temperature and stirred overnight. All solvents were removed *in vacuo* and desired product was precipitated by CH_2Cl_2, MeOH and Et_2O.

5.2.7) Deprotection of nitro group (-NO_2) from guanidine side chain by catalytic hydrogenolysis.

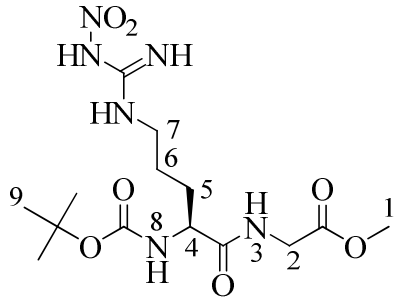
To a stirred solution of peptide (1.0 mmol) in MeOH: AcOH (4 mL of a 3: 1 mixture, required for 1.0 mmol preparation) was added 10% Pd/C (10% wt. of substrate) and hydrogenated by the use of hydrogen balloon at room temperature. Reaction mixture was stirred overnight or until disappearance of the starting material and then filtered through celite^{TM}. The filter cake was washed with MeOH (50 mL, required for 1.0 mmol preparation). The filtrate was concentrated *in vacuo* and trituration with Et_2O.

5.3) EXPERIMENTAL PART FOR CHAPTER 2

5.3.1) Synthesis of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 64.

N-Boc-*L*-Arg(NO_2)-Gly-OMe 56

Compound **56** was prepared following general procedure section 5.2.1 (6.3 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10% MeOH) and precipitated from CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.92 g, 78 %).



m.p.: 56 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.22 (1NH-3, t, *J* = 6.0 Hz); 6.88 (1NH-8, d, *J* = 8.0 Hz); 3.94 (1H-4, m); 3.83 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.61 (3H-1, s); 3.13 (2H-7, apparent d, *J* = 6.0 Hz); 1.50-1.65 (2H-5, 2H-6, m); 1.37 (9H-9, s). There are 3 x N-H protons of arginine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 172.96 (C), 170.66 (C), 159.74 (C), 155.73 (C), 78.57 ($C(CH_3)_3$), 53.16 (CH-4), 52.10 (OCH_3-1), 40.99 (CH_2 x 2), 40.75 (CH_2), 29.63 (CH_2), 28.64 ($C(CH_3)_3$).

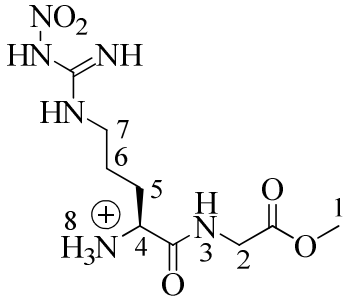
IR (cm^{-1}): 3301 (N-H, br), 2975 (C-H, br), 1756 (O=C, m), 1660 (O=C, s), 1529 (N=O, m), 1368 (N-H, C-N, m), 1250 (C-N, N-H, m).

LRMS (ES$^+$): 413 [(M + Na)$^+$, 100%], 803 [(2M + Na)$^+$, 9%].

HRMS (ES$^+$): $C_{14}H_{26}N_6NaO_7$ (M + Na)$^+$: calculated 413.1755, found 413.1761.

H-*L*-Arg(NO_2)-Gly-OMe 57

Compound **57** was prepared following general procedure section 5.2.2 (1.0 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (1.11 g, 83%).



m.p.: 112 ºC

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.93 (1NH-3, t, *J* = 6.0 Hz); 8.22 (1NH_3-8, br); 7.95-7.99 (2NH, br); 3.98 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.85 (1H-4, m); 3.65 (3H-1, s); 3.18 (2H-7, apparent d, *J* = 6.0 Hz); 1.75 (2H-5, m); 1.57 (2H-6, m). There is one N-H proton of arginine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 170.23 (C), 169.57 (C), 159.75 (C), 52.31 (CH-4), 52.01 (OCH_3-1), 41.14 (CH_2), 41.03 (CH_2), 40.39 (CH_2), 28.81 (CH_2).

IR (cm^{-1}): 2939 (C-H, br), 1736 (O=C, s), 1630 (O=C, s), 1366 (N-H, C-N, m), 1266 (C-N, N-H, s).

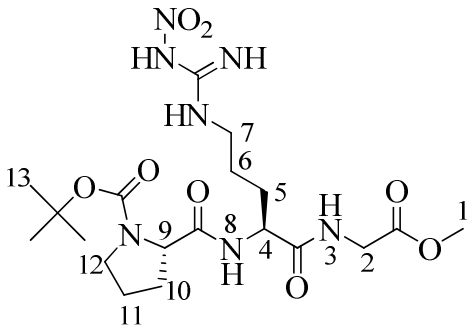
LRMS (ES$^+$): 291 [(M + H)$^+$, 100%], 313 [(M+Na)$^+$, 69%].

HRMS (ES$^+$): $C_9H_{16}N_6O_5$ (M + H)$^+$: calculated 291.1411, found 291.1413.

***N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-OMe 58**

Compound **58** was prepared following general procedure section 5.2.1 (5.3 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10%

MeOH) and precipitated from CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.73 g, 67 % yield).



m.p.: 65 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.53 (1NH, br); 8.37 (1NH-3, br); 7.92 (1NH-8, d, *J* = 8.0 Hz); 4.32 (1H-4, m); 4.14 (1H-9, apparent d, *J* = 8.0 Hz); 3.84 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.62 (3H-1, s); 3.28 (2H-12, t, *J* = 7.0 Hz); 3.16 (2H-7, apparent d, *J* = 6.0 Hz); 2.08 (2H-10, m); 1.77 (2H-11, m); 1.67 (2H-5, m); 1.55 (2H-6, m); 1.30 and 1.37 (9H-13, 2 x s, there are two singlets from rotamers). There are 2 x N-H protons of arginine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.29 (C), 172.86 (C), 171.05 (C), 160.22 (C), 154.27 (C), 79.38 (*C*$(CH_3)_3$), 60.32 (CH), 52.68 (CH), 52.63 (OCH_3), 47.44 (CH_2), 41.44 (CH_2), 41.16 (CH_2), 31.94 (CH_2), 30.65 (CH_2), 24.88 (CH_2), 23.99 (CH_2). There are the extra peaks from the rotamers at 154.83 (C), 79.74 (*C*$(CH_3)_3$), 47.65 (CH_2), 30.44 (CH_2), 30.09 (CH_2) and 29.04 (C(*C*$H_3)_3$) included.

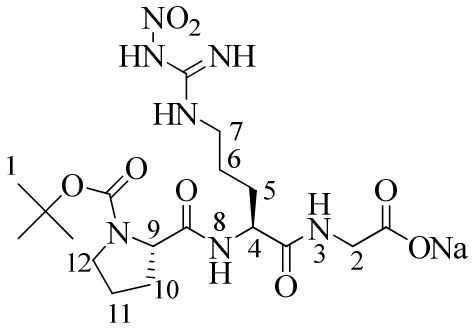
IR (cm^{-1}): 3289 (N-H, br), 2970 (C-H, br), 1739 (C=O, s), 1626 (C=O, s), 1538 (N=O, m), 1366 (N-H, C-N, s), 1229 (C-N, N-H, s).

LRMS (ES$^+$): 510 [(M + Na)$^+$, 100%], 997 [(2M+Na)$^+$, 17%].

HRMS (ES$^+$): $C_{19}H_{33}N_7NaO_8$ (M + Na)$^+$: calculated 510.2283, found 510.2278.

N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-ONa 59

Compound **59** was prepared following general procedure section 5.2.4 (1.0 mmol). The title product was obtained as a white solid and was use in the next step without further purification (0.25 g, 53%).



m.p.: 113 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.51 (1NH, br); 8.03 (1NH-3, br); 7.95 (1NH-8, d, *J* = 8.0 Hz); 7.92 (1NH, br); 4.30 (1H-4, m); 4.13 (1H-9, dd, *J* = 4.0, 8.0 Hz); 3.68 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.27 (2H-12, m); 3.15 (2H-7, apparent d, *J* = 6.0 Hz); 2.07 (2H-10, m); 1.79 (2H-11, m); 1.74 (2H-5, m); 1.53 (2H-6, m); 1.31 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers). There is one N-H proton of arginine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 179.22 (C), 171.51 (C), 170.58 (C), 158.45 (C), 152.49 (C), 77.59 (*C*(CH$_3$)3), 59.84 (CH), 52.32 (CH), 46.95 (CH$_2$), 41.79 (CH$_2$), 40.66 (CH$_2$), 31.46 (CH$_2$), 29.86 (CH$_2$), 28.56 (CH$_2$), 28.43 (C(*C*H$_3$)$_3$), 24.38 (CH$_2$).

There are extra peaks from rotamers at 77.92 (*C*(CH$_3$)$_3$), 47.15 (CH$_2$), 40.40 (CH$_2$), 40.19 (CH$_2$), 31.98 (CH$_2$), 30.17 (CH$_2$), 28.56 (C(*C*H$_3$)$_3$).

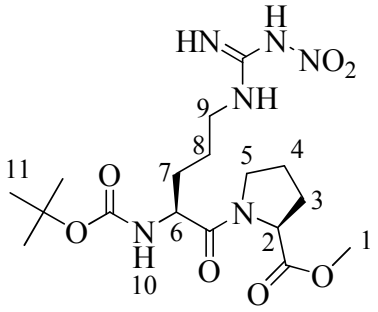
IR (cm^{-1}): 3220 (N-H, br), 2936 (C-H, br), 1742 (C=O, m), 1629 (C=O, s), 1541 (N=O, m), 1367 (N-H, C-N, s), 1261 (C-N, N-H, s).

LRMS (ES^{+}): 518 [(M + Na)$^{+}$, 100%], 1041 [(2M+Na)$^{+}$, 20%].

HRMS (ES^{+}): $C_{18}H_{30}N_7Na_2O_8$ (M + Na)$^{+}$: calculated 518.1946, found 518.1939.

N-Boc-*L*-Arg(NO$_2$)-*L*-Pro-OMe 60

Compound **60** was prepared following general procedure section 5.2.1 (6.3 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10% MeOH) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.63 g, 60 %).



m.p.: 55 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 7.53-7.59 (2 x NH, br); 5.63 (1NH-10, d, *J* = 8.0 Hz); 4.31 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.18 (1H-6, m); 3.60 (3H-1, s); 3.55 (2H-5, m); 3.13 (2H-9, apparent d, *J* = 6.0 Hz); 2.25 (2H-3, apparent dt, *J* = 8.0, 8.0, 12.0 Hz); 1.92 (2H-4, apparent p, *J* = 8.0 Hz); 1.81 (2H-8, dt, *J* = 8.0, 8.0, 12.0 Hz); 1.55 (2H-7, m); 1.36 (9H-11, s). There is one N-H proton of arginine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.24 (C), 171.51 (C), 160.30 (C), 156.31 (C), 78.99 ($C(CH_3)_3$), 59.38 (CH), 52.66 (OCH_3-1), 52.45 (CH), 47.32 (CH_2), 41.33 (CH_2), 31.84 (CH_2), 29.44 (CH_2) 29.14 ($C(CH_3)_3$), 28.88 (CH_2), 25.60 (CH_2).

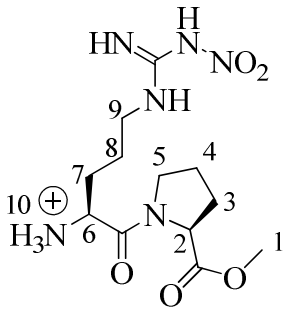
IR (cm^{-1}): 3305 (N-H, br), 2978 (C-H, br), 1744 (C=O, m), 1628 (O=C, s), 1533 (N=O, m), 1366 (N-H, C-N, m), 1254 (C-N, N-H, s).

LRMS (ES^{+}): 453 [$(M + Na)^{+}$, 100%], 883 [$(2M + Na)^{+}$, 52%].

HRMS (ES^{+}): $C_{17}H_{30}N_6NaO_7$ $(M + Na)^{+}$: calculated 453.2068, found 453.2070.

H-*L*-Arg(NO_2)-*L*-Pro-OMe 61

Compound **61** was prepared following general procedure section 5.2.2 (4.7 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (1.37 g, 85%).

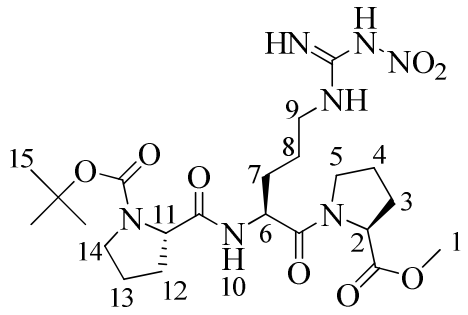


m.p.: 108 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.59 (1NH, br); 8.32 (1NH$_3$-10, br); 8.04-8.05 (2NH, br); 4.37 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.19 (1H-6, m); 3.80 (2H-5, m); 3.63 and 3.75 (3H-1, 2 x s, there are two singlets from rotamers); 3.17 (2H-9, m); 2.25 (2H-3, apparent dt, *J* = 8.0, 8.0, 12.0 Hz); 1.93 (2H-4, apparent p, *J* = 8.0 Hz); 1.82 (2H-8, m); 1.63 (2H-7, m).
^{13}C NMR (100 MHz, d^6-DMSO): δ 172.65 (C), 168.24 (C), 160.26 (C), 59.68 (CH), 52.89 (OCH_3-1), 51.30 (CH), 47.67 (CH_2), 41.73 (CH_2), 41.02 (CH_2), 29.48 (CH_2), 28.09 (CH_2), 25.68 (CH_2). There are extra peaks from rotamers at 52.49 (OCH_3-1) and 28.18 (CH_2) included.
IR (cm^{-1}): 2875 (C-H, br), 1742 (C=O, m), 1630 (C=O, s), 1595 (N=O, m), 1365 (N-H, C-N, m), 1265 (C-N, N-H, s).
LRMS (ES$^+$): 331 [(M + H)$^+$, 100%], 353 [(M + Na)$^+$, 53%], 683 [(2M+Na)$^+$, 15%].
HRMS (ES$^+$): $C_{12}H_{23}N_6O_5$ (M + H)$^+$: calculated 331.1724, found 331.1728.

N-Boc-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 62

Compound **62** was prepared following general procedure section 5.2.1 (27.8 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10% MeOH) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.75 g, 60 %).



m.p.: 71 ˚C
^{1}H NMR (400 MHz, d^6-DMSO): δ 8.54 (1NH, br); 8.04 (1NH-10, d, *J* = 8.0 Hz); 4.51 (1H-6, m); 4.30 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.12 (1H-11, m); 3.69 (2H-5, m); 3.60 (3H-1, s); 3.34 (1H-14, m); 3.16 (2H-9, m); 1.54-2.19 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, m); 1.30 and 1.37 (9H-15, 2 x s, there are two singlets from rotamers). There are 2 x N-H protons of arginine side chain missing from the ^{1}H-spectrum.
^{13}C NMR (100 MHz, d^6-DMSO): δ 173.14 (C), 170.86 (C), 160.29 (C), 154.55 (C), 154.22 (C), 79.33 ($C(CH_3)_3$), 60.24 (CH), 59.40 (CH), 52.69 (OCH_3-1), 50.72 (CH), 47.42 (CH_2),

41.28 (CH_2), 31.92 (CH_2), 30.69 (CH2), 29.49 (CH_2), 29.22 (CH_2), 28.92 (C(CH_3)$_3$), 25.56 (CH_2), 24.81 (CH_2), 23.98 (CH_2). There are the extra peaks from the rotamers at 173.33 (C), 170.76 (C), 79.52 (*C*(CH_3)$_3$), 60.11 (CH), 47.60 (CH_2), 31.76 (CH_2) and 29.03 (C(*C*H_3)$_3$) included.

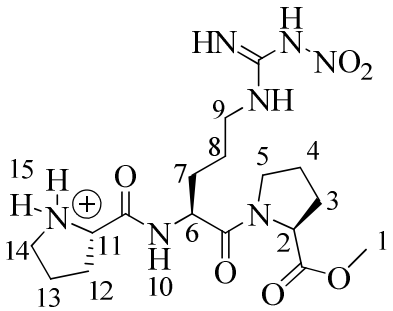
IR (cm^{-1}): 3304 (N-H, br), 2975 (C-H, br), 1744 (C=O, s), 1625 (C=O, s), 1536 (N=O, m), 1393 (N-H, C-N, s), 1254 (C-N, N-H, s).

LRMS (ES$^+$): 550 [(M + Na)$^+$, 100%], 1077 [(2M + Na)$^+$, 33%].

HRMS (ES$^+$): $C_{22}H_{37}N_7NaO_8$ (M + Na)$^+$: calculated 550.2596, found 550.2587.

H-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 63

Compound **63** was prepared following general procedure section 5.2.2 (2.0 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.86 g, 80%).



m.p.: 108 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 10.08 (1NH, br); 8.84 (1NH-10, d, *J* = 8.0 Hz); 8.49 (1NH_2-15, br); 7.96-7.98 (2NH, br); 4.51 (1H-6, m); 4.30 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.22 (1H-11, m); 3.64 (2H-5, t, *J* = 4.0 Hz); 3.60 (3H-1, s); 3.16-3.20 (2H-14, 2H-9, m); 1.59-2.31 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, m). There are 2 x N-H protons of arginine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.21 (C), 170.49 (C), 169.16 (C), 160.34 (C), 59.64 (CH), 59.50 (CH), 52.88 (OCH_3-1), 51.85 (CH), 47.65 (CH_2), 46.61 (CH_2), 41.45 (CH_2), 31.92 (CH_2), 30.70 (CH_2), 29.60 (CH_2), 28.80 (CH_2), 25.79 (CH_2), 24.57 (CH_2). There are the extra peaks from rotamers at 174.13 (C), 170.30 (C), 47.60 (CH_2), 29.66 (CH_2) and 25.70 (CH_2) included.

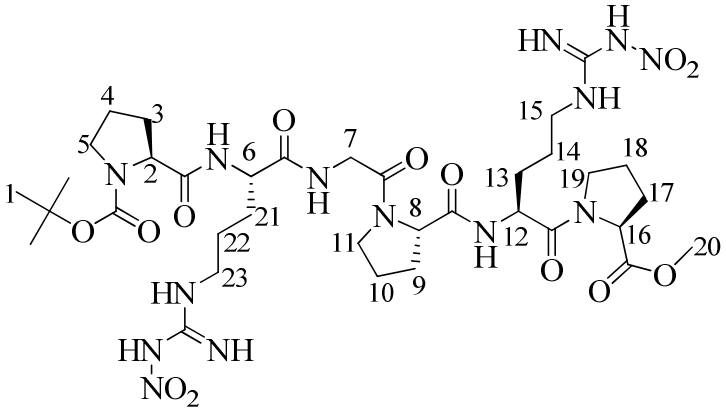
IR (cm^{-1}): 3292 (N-H, br), 2970 (C-H, br), 1738 (C=O, s), 1626 (C=O, m), 1539 (N=O, m), 1365 (N-H, C-N, s), 1229 (C-N, N-H, s).

LRMS (ES$^+$): 428 [(M + H)$^+$, 100%], 450 [(M+Na)$^+$, 24%], 855 [(2M+H)$^+$, 7%].

HRMS (ES$^+$): $C_{17}H_{29}N_7NaO_6$ (M + Na)$^+$: calculated 450.2072, found 450.2071.

N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 64

Compound **64** was prepared following general procedure section 5.2.1 (0.3 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated from CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.16 g, 62 %).



m.p.: 82 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.52 (1NH, br); 8.41 (1NH, d, *J* = 8.0 Hz); 8.12 (1NH, d, *J* = 8.0 Hz); 7.94-7.97 (5NH, br); 4.52-3.88 (1H-2, 1H-6, 2H-7, 1H-8, 1H-12, 1H-16, m); 3.60 (3H-20, s); 3.15-3.69 (2H-5, 2H-23, 2H-11, 2H-15, 2H-19, m); 1.54-2.21 (2H-3, 2H-4, 2H-21, 2H-22, 2H-9, 2H-10, 2H-13, 2H-14, 2H-17, 2H-18, m); 1.31 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers). There is one N-H proton missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.29 (C), 173.14 (C), 172.96 (C), 172.38 (C), 172.28 (C), 170.71 (C), 167.67 (C), 167.62 (C), 160.22 (C), 77.26 (*C*(CH_3)$_3$), 60.14 (CH), 59.40 (CH), 52.82 (CH), 52.62 (OCH_3-20), 52.37 (CH), 50.82 (CH), 47.34 (CH_2), 46.75 (CH_2), 42.15 (CH_2), 41.20 (CH_2), 41.14 (CH_2), 41.04 (CH_2), 40.25 (CH_2), 38.53 (CH_2), 36.94 (CH_2), 33.34 (CH_2), 30.09 (CH_2), 29.45 (CH_2), 28.89 (C(CH_3)$_3$), 29.08 (CH_2), 25.52 (CH_2), 25.06 (CH_2), 23.94 (CH_2). There is the extra peak from rotamers at 77.53 (*C*(CH_3)$_3$) included.

IR (cm^{-1}): 3295 (N-H, br), 2970 (C-H, br), 1740 (C=O, m), 1626 (C=O, s), 1533 (N=O, m), 1366 (N-H, C-N, s), 12577 (C-N, N-H, s).

LRMS (ES$^+$): 464 [(M+2Na)$^{2+}$, 30%], 905 [(M+Na)$^+$, 100%].

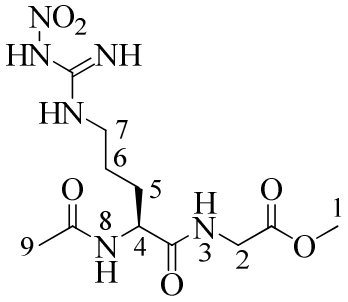
HRMS (ES$^+$): $C_{35}H_{58}N_{14}NaO_{13}$ $(M + Na)^+$: calculated 905.4200, found 905.4216.

5.3.2) Model study of side chain modification *L*-Arg(NO_2)-Gly series.

Side chain modification of simple dipeptide, *N*-Boc-*L*-Arg(NO_2)-Gly-OMe which was *N*-Boc deprotection, Acetylation at N-terminal, amidolysis at C-terminal and removal of nitro protecting group by catalytic hydrogenolysis.

Acetylation of *N*-terminus of peptide: *N*-Ac-*L*-Arg(NO_2)-Gly-OMe 65

Compound **65** was prepared following general procedure section 5.2.5 (1.0 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2). The product was precipitated from CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.21 g, 62 %).



m.p.: 59 ˚C

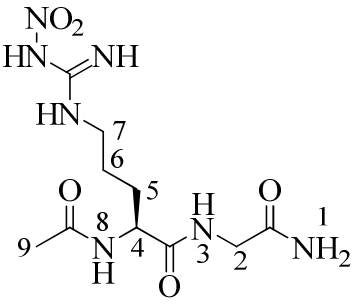
^{1}H NMR (400 MHz, d^6-DMSO): δ 8.37 (1NH-3, t, *J* = 6.0 Hz); 8.06 (1NH-8, d, *J* = 8.0 Hz); 7.92 (1NH, br); 4.27 (1H-4, m); 3.82 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.61 (3H-1, s); 3.13 (2H-7, m); 1.85 (3H-9, s); 1.66 (2H-5, m); 1.51 (2H-6, m). There are 2 x N-H protons of arginine side chain missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.13 (C), 171.11 (C), 170.27 (C), 160.22 (C), 52.86 (CH-4), 52.60 (OCH_3-1), 49.52 (CH_2), 41.48 (CH_2), 41.18 (CH_2), 30.20 (CH_2), 23.45 (CH_3-9). There is extra peak from rotamer at 41.55 (CH_2) included.

IR (cm^{-1}): 3292 (N-H, br), 2952 (C-H, br), 1741 (C=O, m), 1622 (C=O, s), 1533 (N=O, m), 1253 (C-N, N-H, s).

Amide formation of *C*-terminus of the peptide: *N*-Ac-*L*-Arg(NO_2)-Gly-C(O)-NH_2 66.

Compound **66** was prepared following general procedure section 5.2.6 (0.62 mmol). The title compound was obtained as a white solid without further purification (0.19 g, 95 %).



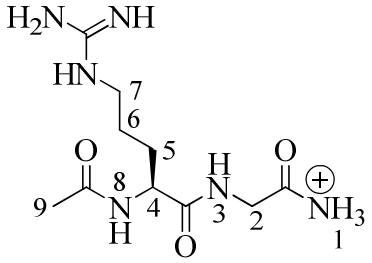
m.p.: 78 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.18 (1NH-3, t, *J* = 6.0 Hz); 8.14 (1NH-8, d, *J* = 8.0 Hz); 7.19 (1NH, br); 7.09 (1NH_2-1, br); 4.14 (1H-4, apparent t, *J* = 8.0 Hz); 3.62 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.14 (2H-7, m); 1.85 (3H-9, s); 1.50-1.70 (2H-5, 2H-6, m). There are 2 x N-H protons of arginine side chain missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 172.76 (C), 171.82 (C), 170.70 (C), 160.20 (C), 53.62 (CH-4), 49.49 (CH_2), 46.38 (CH_2), 42.82 (CH_2), 29.65 (CH_2), 23.40 (CH_3-Ac).

Deprotection of nitro (NO_2) group: *N*-Ac-*L*-Arg(H)-Gly-C(O)-NH_2 67.

Compound **67** was prepared following general procedure section 5.2.7 (0.59 mmol). The title compound was obtained as a white solid without further purification (0.13 g, 78%).



m.p.: 86 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.25 (1NH-3, t, *J* = 6.0 Hz); 8.14 (1NH-8, d, *J* = 8.0 Hz); 7.90 (1NH, br); 7.22 (NH_3-1, br); 7.04 (1NH, br); 4.17 (1H-4, apparent dq, *J* = 8.0, 8.0, 8.0, 28.0 Hz); 3.62 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.17 (2H-7, m); 1.87 (3H-9, s); 1.50-1.70 (2H-4, 2H-5, m). There are 2 x N-H protons of guanidine part missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 181.47 (C), 172.78 (C), 172.06 (C), 169.93 (C), 55.81 (CH), 49.49 (CH_2), 42.83 (CH_2), 42.30 (CH_2), 29.46 (CH_2), 23.40 (CH_3-Ac).

IR (cm^{-1}): 3294 (N-H, br), 2944 (C-H, br), 1654 (C=O, m), 1269 (C-N, N-H, m).

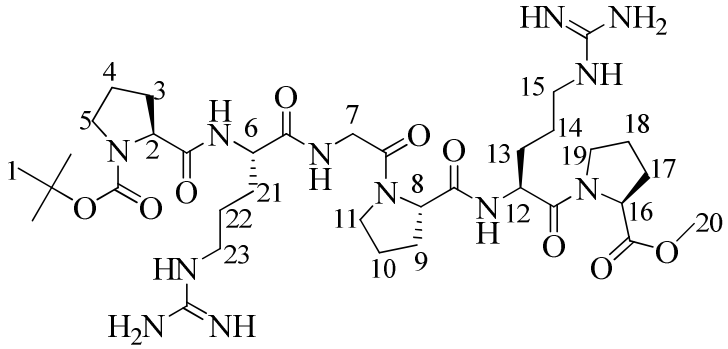
LRMS (ES$^+$): 273 [(M+H)$^+$, 100%].

5.3.3) Structural modification of hexapeptide *L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$) -*L*-Pro.

Modification of the group on fully protected hexapeptides resulted in a series that had only side chain differences.

N-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe 68.

Compound **68** was prepared from *N*-Boc-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **64** (1.0 mmol) by following general procedure section 5.2.7. The title compound was obtained as a white solid without further purification (0.73 g, 92%).



m.p.: 109 ºC

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.63 (1NH, d, *J* = 8.0 Hz); 8.38 (1NH, d, *J* = 8.0 Hz); 8.26 (1NH, d, *J* = 8.0 Hz); 8.20 (1NH, d, *J* = 8.0 Hz); 8.06 (1NH, br); 3.79-4.49 (1H-2, 1H-6, 2H-7, 1H-8, 1H-12, 1H-16, m); 3.60 (3H-20, s); 3.00-4.03 (2H-5, 2H-23, 2H-11, 2H-15, 2H-19, m); 1.48-2.18 (2H-3, 2H-4, 2H-21, 2H-22, 2H-9, 2H-10, 2H-13, 2H-14, 2H-17, 2H-18, m); 1.31 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers). There are 6 x N-H protons missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 177.64 (C), 177.60 (C), 173.23 (C), 172.67 (C), 172.49 (C), 170.89 (C), 170.79 (C), 167.68 (C), 158.57 (C), 79.34 (*C*(CH$_3$)$_3$), 60.09 (CH), 59.41 (CH), 52.68 (OCH$_3$-20), 52.59 (CH), 51.29 (CH), 51.01 (CH), 47.82 (CH$_2$), 47.54 (CH$_2$), 47.33 (CH$_2$), 46.79 (CH$_2$), 44.43 (CH$_2$), 42.14 (CH$_2$), 41.84 (CH$_2$), 33.68 (CH$_2$), 32.01 (CH$_2$), 30.80 (CH$_2$), 30.11 (CH$_2$), 29.94 (CH$_2$), 29.47 (CH$_2$), 29.09 (CH$_2$), 28.96 (C(*C*H$_3$)$_3$), 25.59 (CH$_2$), 25.34 (CH$_2$). There are extra peaks from rotamers at 79.55 (*C*(CH$_3$)$_3$), 32.69 (CH$_2$), 31.95 (CH$_2$), 29.89 (CH$_2$), 28.89 (C(*C*H$_3$)$_3$), 25.71 (CH$_2$) and 25.18 (CH$_2$) included.

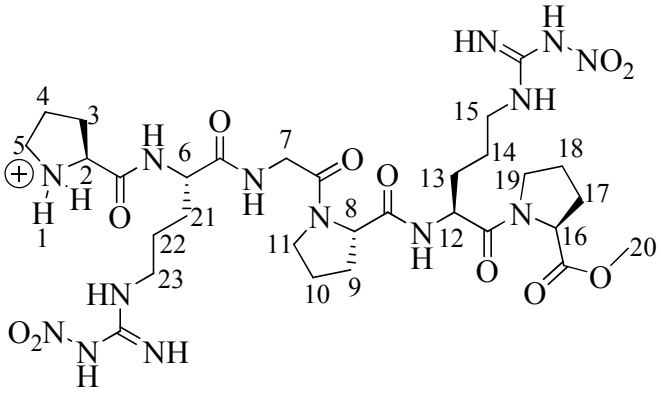
IR (cm^{-1}): 3269 (N-H, br), 2973 (C-H, br), 1742 (C=O, m), 1653 (C=O, s), 1366 (N-H, C-N, m).

LRMS (ES$^+$): 397 [(M+2H)$^{2+}$, 100%], 793 [(M+H)$^+$, 22%].

HRMS (ES$^+$): $C_{35}H_{62}N_{12}O_9$ (M + 2H)$^{2+}$: calculated 397.2376, found 397.2377.

H-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 69.

Compound **69** was prepared from *N*-Boc-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe **64** (1.0 mmol) by following general procedure section 5.2.3. The title compound was obtained as a white solid without further purification (0.74 g, 95%).



m.p.: 101 ºC

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.73 (1NH, d, *J* = 8.0 Hz); 8.52 (1NH, t, *J* = 8.0 Hz); 8.42 (1NH, t, *J* = 4.0 Hz); 8.37 (1NH, t, *J* = 4.0 Hz); 8.15 (1NH, t, *J* = 4.0 Hz); 8.13 (1NH, t, *J* = 4 Hz); 7.87-7.96 (3NH, br); 3.80-4.48 (1H-2, 1H-6, 2H-7, 1H-8, 1H-12, 1H-16, m); 3.60 (3H-20, s); 3.15-3.97 (2H-5, 2H-23, 2H-11, 2H-15, 2H-19, m); 1.47-2.32 (2H-3, 2H-4, 2H-21, 2H-22, 2H-9, 2H-10, 2H-13, 2H-14, 2H-17, 2H-18, m). There are 2 x N-H protons of peptide chain or guanidine part missing from the ^{1}H-spectrum.

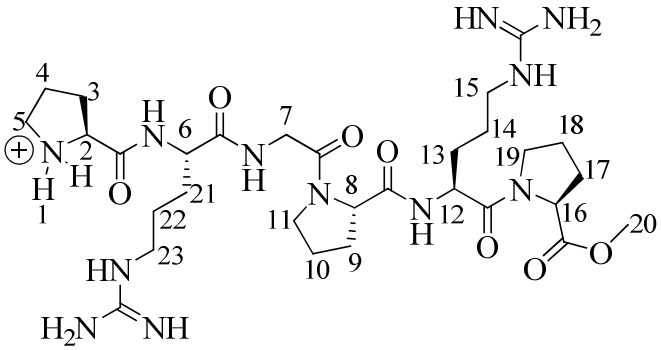
IR (cm^{-1}): 3280 (N-H, br), 2953 (C-H, br), 1739 (C=O, m), 1627 (C=O, s), 1538 (N=O, m), 1260 (C-N, N-H, s).

LRMS (ES$^+$): 783 [(M+H)$^+$, 100%].

HRMS (ES$^+$): $C_{30}H_{61}N_{14}O_{11}$ (M + H)$^+$: calculated 783.3843, found 783.3844.

H-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe 70.

Compound **70** was prepared from *N*-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe **68** (0.34 mmol) by following general procedure section 5.2.3. The title compound was obtained as a white solid without further purification (0.23 g, 98%).



m.p.: 111 ºC

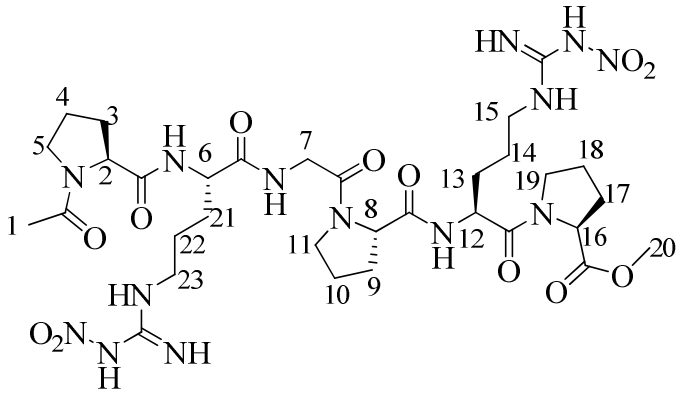
IR (cm^{-1}): 3274 (N-H, br), 2956 (C-H, br), 1741 (C=O, m), 1626 (C=O, s), 1266 (C-N, N-H, s).

LRMS (ES$^+$): 347 [$(M+2H)^{2+}$, %], 693 [$(M+H)^+$, %].

HRMS (ES$^+$): $C_{30}H_{54}N_{12}O_7$ $(M + 2H)^{2+}$: calculated 347.2114, found 347.2111.

***N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 71.**

Compound **71** was prepared from H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **69** (0.73 mmol) by following general procedure section 5.2.5. The title compound was obtained as a white solid without further purification (0.38 g, 63%).



m.p.: 65 ºC

^{1}H NMR (400 MHz, d^6-DMSO): δ 11.14 (1NH, br); 9.36 (1NH, br); 8.41 (1NH, d, *J* = 8.0 Hz); 8.24 (1NH, d, *J* = 8.0 Hz); 8.10 (1NH, d, *J* = 8.0 Hz); 8.01 (1NH, t, *J* = 4.0 Hz); 7.83 (1NH, t, *J* = 4.0 Hz); 3.91-4.53 (1H-2, 1H-6, 2H-7, 1H-8, 1H-12, 1H-16, m); 3.59 (3H-20, s); .13-3.70 (2H-5, 2H-23, 2H-11, 2H-15, 2H-19, m); 1.97 (3H-1, s); 1.47-2.03 (2H-3, 2H-4, 2H-21, 2H-22, 2H-9, 2H-10, 2H-13, 2H-14, 2H-17, 2H-18, m). There are 2 x N-H protons of peptide chain and guanidine side chain missing from the ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 180.89 (C), 180.42 (C), 173.13 (C), 172.65 (C), 172.40 (C), 170.62 (C), 170.49 (C), 167.52 (C), 155.10 (C), 60.13 (CH), 60.08 (CH), 59.47 (CH), 55.75 (CH), 52.64 (OCH_3-20), 50.83 (CH), 48.37 (CH_2), 47.65 (CH_2), 46.97 (CH_2), 46.22 (CH_2), 42.11 (CH_2), 37.73 (CH_2), 35.53 (CH_2), 32.66 (CH_2), 30.25 (CH_2), 29.49 (CH_2), 25.56 (CH_2), 25.27 (CH_2), 25.09 (CH_2), 23.20 (CH_3-1), 23.17 (CH_2), 22.91 (CH_2). There is one CH_2-peak missing because the overlapping of peaks from spectrum.

IR (cm^{-1}): 3293 (N-H, br), 2954 (C-H, br), 1742 (C=O, m), 1627 (C=O, s), 1539 (N=O, m), 1260 (C-N, N-H, s).

LRMS (ES$^+$): 847 [(M+Na)$^+$, 100%], 1672 [(2M+Na)$^+$, 10%].

LCMS (ES$^+$): 825 [(M+H)$^{2+}$, 30%], 847 [(M+Na)$^+$, 100%].

HRMS (ES$^+$): $C_{32}H_{52}N_{14}NaO_{12}$ (M + H)$^+$: calculated 847.3781, found 847.3774.

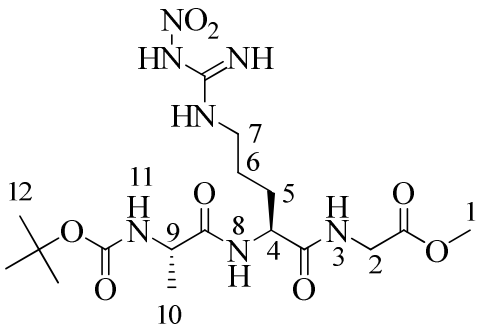
5.3.4) Solution phase peptide synthesis of *L*-alanine (Ala) scanning peptides.

Alanine scanning compounds were synthesized by replace each position of amino acid in peptide chain with alanine (Ala). The synthesized was carried on by solution phase peptide synthesis and general procedures were achieved in section 5.2.

a) Synthesis of Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 76.

N-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-OMe 72

Compound **72** was prepared following general procedure section 5.2.1 (1.1 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10% MeOH) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.35 g, 70 %).



m.p.: 50 ˚C

^{1}H NMR (300 MHz, d^6-DMSO): δ 8.51 (1NH, br); 8.38 (1NH-3, t, *J* = 6.0 Hz); 7.94 (1NH, br); 7.82 (1NH-8, d, *J* = 8.0 Hz); 6.98 (NH-11, d, *J* = 8.0 Hz); 4.32 (1H-4, m); 4.02 (1H-9, m); 3.85 (2H-2, apparent t, *J* = 6.0 Hz); 3.61 (3H-1, s); 3.15 (2H-7, m) 1.68 (2H-5, m); 1.65 (2H-6, m); 1.36 (9H-12, s); 1.15-1.17 (3H-10, d, *J* = 8.0 Hz). There is one N-H proton missing from the ^{1}H-spectrum.

^{13}C NMR (75 MHz, d^6-DMSO): δ 173.63 (C), 172.83 (C), 171.04 (C), 160.23 (C), 156.08 (C), 79.13 (*C*(CH$_3$)$_3$), 52.67 (OCH$_3$-1), 52.63 (CH), 50.82 (CH), 41.51 (CH$_2$), 41.21 (CH$_2$), 30.57 (CH$_2$), 29.13 (C(*C*H$_3$)$_3$), 18.93 (CH$_3$-10). There is one CH$_2$ peak missing because overlapping of CH$_2$ peaks from ^{13}C-spectrum.

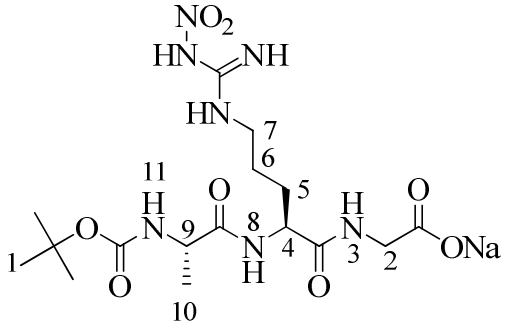
IR (cm^{-1}): 3297 (N-H, br), 2980 (C-H, br), 1748 (C=O, m), 1653 (C=O, s), 1522 (N=O, s), 1367 (N-H, C-N, m), 1251 (C-N, N-H, s).

LRMS (ES$^+$): 484 [(M + Na)$^+$, 100%], 945 [(2M + Na)$^+$, 18%], 1406 [(3M+Na)$^+$, 5%].

HRMS (ES$^+$): $C_{17}H_{31}N_7NaO_8$ (M + Na)$^+$: calculated 484.2126, found 484.2123.

N-Boc-*L*-Ala-*L*-Arg(NO$_2$)-Gly-ONa 73

Compound **73** was prepared following general procedure section 5.2.4 (1.0 mmol). The title product was obtained as a white solid and was use in the next step without further purification (0.54 g, 75%).



m.p.: 102 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.21 (1NH-3, br); 7.79 (1NH-8, d, *J* = 8.0 Hz); 6.97 (1NH-11, d, *J* = 8.0 Hz); 4.31 (1H-4, m); 4.01 (1H-9, m); 3.72 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.17 (2H-7, m); 1.69 (2H-5, m); 1.52 (2H-6, m); 1.36 (9H-1, s); 1.16 and 1.18 (3H-10, 2 x d, *J* = 8.0 Hz, there are two doublets from rotomers). There are 3 x NH protons from guanidine part missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.48 (C), 172.37 (C), 171.79 (C), 160.29 (C), 155.87 (C), 78.93 (*C*(CH$_3$)$_3$), 52.07 (CH), 50.28 (CH), 41.10 (CH$_2$), 40.67 (CH$_2$), 30.06 (CH$_2$),

28.60 (C(CH_3)$_3$), 24.50 (CH_2), 18.39 (CH_3-10). There are extra peaks from rotamer at 60.21 (CH), 49.04 (CH), 21.50 (CH_2) and 21.20 (CH_2) included.

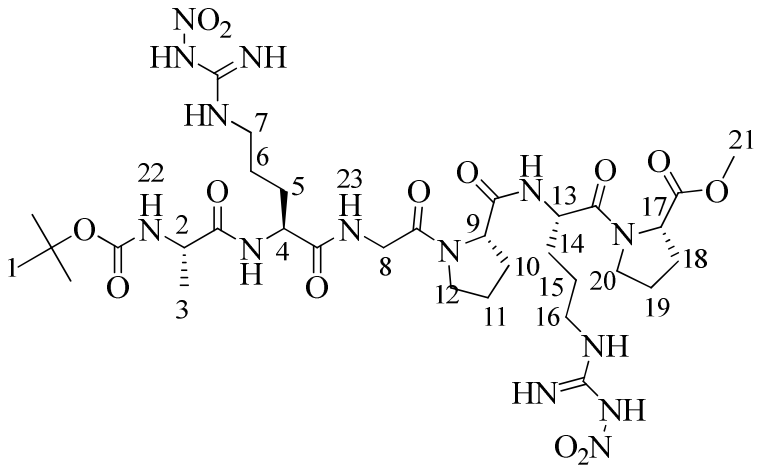
IR (cm^{-1}): 3296 (N-H, br), 2979 (C-H, s), 1633 (O=C, s), 1525 (N=O, s), 1367 (N-H, C-N, s), 1249 (C-N, N-H, s).

LRMS (ES$^+$): 470 [(M + H)$^+$, 100%].

HRMS (ES$^+$): $C_{16}H_{29}N_7NaO_8$ (M + Na)$^+$: calculated 470.1970, found 470.1960.

N-Boc-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 74

Compound **74** was prepared following general procedure section 5.2.1 (0.3 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O to yield the title compound as a white solid (0.05 g, 54 %).



m.p.: 78 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.51 (1NH, br); 8.41 (1NH, d, *J* = 8.0 Hz); 8.12 (1NH, d, *J* = 8.0 Hz); 7.98 (1NH-23, t, *J* = 6.0 Hz); 7.84-7.95 (4NH, br); 7.45 (1NH, br); 6.94 (1NH-22, d, *J* = 8.0 Hz); 4.27-4.54 (1H-4, 1H-9, 1H-13, 1H-17, m); 4.00 (1H-2, m); 3.83 (2H-8, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.60 (3H-21, s); 3.11-3.64 (2H-7, 2H-12, 2H-16, 2H-20, m); 1.52-2.21 (2H-5, 2H-6, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.27 and 1.36 (9H-1, 2 x s, there are two singlets from rotamers); 1.16-1.18 (3H-3, d, *J* = 8.0 Hz).

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.19 (C), 172.48 (C), 172.38 (C), 172.34 (C), 170.77 (C), 167.75 (C), 167.67 (C), 160.26 (C), 156.00 (C), 79.05 (*C*(CH_3)$_{3)}$, 60.21 (CH), 59.46 (CH), 54.38 (CH), 52.68 (OCH_3-21), 51.18 (CH), 50.91 (CH), 50.64 (CH_2), 47.41 (CH_2), 46.82 (CH_2), 42.66 (CH_2), 42.19 (CH_2), 41.18 (CH_2), 32.73 (CH_2), 30.52 (CH_2),

29.10 ($C(CH_3)_3$), 25.58 (CH_2), 25.12 (CH_2), 23.00 (CH_2), 18.97 (CH_3-3), 17.62 (CH_2). There are extra peaks from rotamers at 47.72 (CH_2), 30.15 (CH_2), 29.51 ($C(CH_3)_3$) and HOBt at 125.09 (CH), 119.88 (CH), 119.68 (CH), 116.69 (CH) and 110.83 (CH) included.

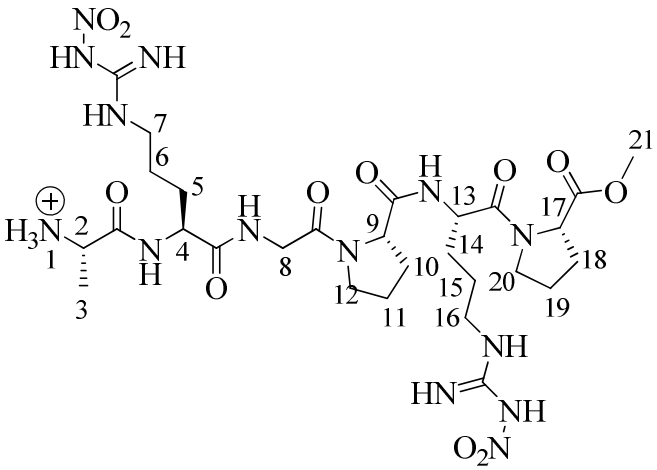
IR (cm^{-1}): 3283 (N-H, br), 2970 (C-H, br), 1739 (O=C-O, m), 1624 (O=C, s), 1541 (N=O, m), 1365 (N-H, C-N, m), 1255 (C-N, N-H, s).

LRMS (ES^+): 879 [$(M+Na)^+$, 100%].

HRMS (ES^+): $C_{33}H_{56}N_{14}NaO_{13}$ $(M + Na)^+$: calculated 879.4043, found 879.4051.

H-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 75

Compound **75** was prepared following general procedure section 5.2.3 (0.12 mmol). The title compound as a white solid and could be used in the next step without further purification (0.91 g, 95%).



m.p.: 87 ˚C

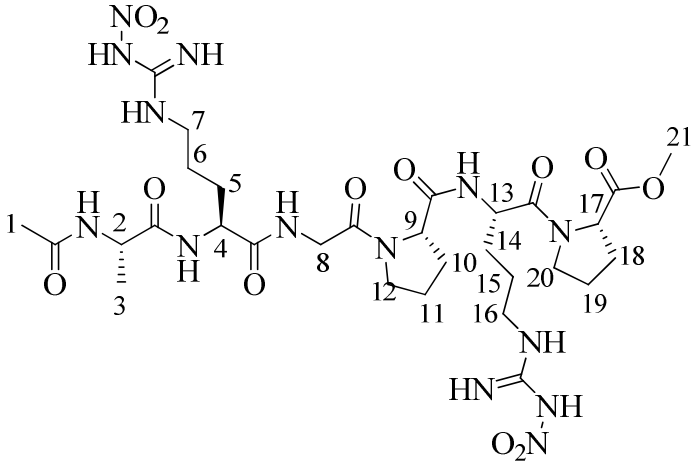
^{1}H NMR (400 MHz, d^6-DMSO): δ 8.57 (1NH, d, *J* = 8.0 Hz); 8.54 (1NH, br); 8.42 (1NH, d, *J* = 8.0 Hz); 8.14 (1NH, d, *J* = 8.0 Hz); 8.07-8.09 (6NH, br); 4.27-4.53 (1H-4, 1H-9, 1H-13, 1H-17, m); 4.00 (1H-2, m); 3.88 (2H-8, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.60 (3H-21, s); 3.16-3.70 (2H-7, 2H-12, 2H-16, 2H-20, m); 1.55-2.21 (2H-5, 2H-6, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.33-1.35 (3H-3, d, *J* = 8.0 Hz). There are 2 x N-H protons missing from 1H-spectrum.

LRMS (ES^+): 390 [$(M+H+Na)^{2+}$, 7%], 757 [$(M+H)^+$, 35%], 779 [$(M+Na)^+$, 30%].

HRMS (ES^+): $C_{28}H_{49}N_{14}O_{11}$ $(M + H)^+$: calculated 757.3700, found 757.3690.

N-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 76

Compound **76** was prepared following general procedure section 5.2.5 (0.12 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2). The product was precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.06 g, 65%).



m.p.: 64 ˚C

^{1}H NMR (400 MHz, $CDCl_3$): δ 11.76 (1NH, d, *J* = 12.0 Hz); 9.41 (1NH, br); 7.60-8.23 (7NH, br); 3.73-4.63 (1H-2, 1H-4, 2H-8, 1H-9, 1H-13, 1H-17, m); 3.62 (3H-21, s); 3.05-3.66 (2H-7, 2H-12, 2H-16, 2H-20, m); 2.10 (3H-1, s); 1.64-2.25 (2H-5, 2H-6, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.34-1.36 (3H-3, d, *J* = 8.0 Hz).

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.47 (C), 172.60 (C), 172.25 (C), 171.60 (C), 171.40 (C), 170.54 (C), 168.22 (C), 159.18 (C), 154.82 (C), 60.43 (CH), 59.04 (CH), 52.35 (CH), 52.32 (OCH_3-21), 50.53 (CH), 49.52 (CH), 47.22 (CH_2), 46.77 (CH_2), 45.90 (CH_2), 42.12 (CH_2), 41.49 (CH_2), 40.86 (CH_2), 30.89 (CH_2), 30.30 (CH_2), 28.96 (CH_2), 25.29 (CH_2), 24.93 (CH_3-Ac), 24.74 (CH_2), 24.48 (CH_2), 22.81 (CH_2), 18.01 (CH_3-3). There are extra peaks from rotamers at 172.16 (C), 157.45 (C), 154.89 (C), 34.20 (CH_2), 29.28 (CH_2), 21.15 (CH_2) and 17.81 (CH_3-3) included.

IR (cm^{-1}): 3273 (N-H, br), 2947 (C-H, br), 1738 (C=O, m), 1633 (C=O, s), 1539 (N=O, m), 1373 (N-H, C-N, m), 1272 (C-N, N-H, s).

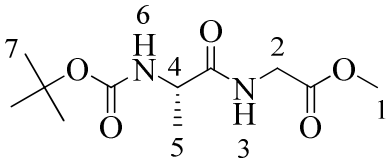
LRMS (ES$^+$): 419 [$(M+Na+NH_4)^{2+}$, 3%], 422 [$(M+2Na)^{2+}$, 31%], 799 [$(M+H)^+$, 2%], 821 [$(M+Na)^+$, 100%], 1619 [$(2M+Na)^+$, 3%].

HRMS (ES$^+$): $C_{30}H_{50}N_{14}NaO_{12}$ $(M + Na)^+$: calculated 821.3625, found 821.3635.

b) Synthesis of Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 83.

***N*-Boc-*L*-Ala-Gly-OMe 77**

Compound **77** was prepared following general procedure section 5.2.1 (1.1 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 2% MeOH/CH_2Cl_2) and yielded the title compound as colourless oil (0.58 g, 42 %).



^{1}H NMR (400 MHz, d^6-DMSO): δ 8.21 (1NH-3, br); 7.08 and 6.95 (1NH-6, 2 x br, there are two broad N-H peaks from rotamers); 4.06 (1H-4, m); 3.87 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.67 (3H-1, s); 1.42 (9H-7, s); 1.23 and 1.26 (3H-5, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.28 (C), 170.24 (C), 154.99 (C), 77.99 ($C(CH_3)_3$), 51.62 (OCH_3-1), 49.46 (CH-4), 40.51 (CH_2-2), 28.19 ($C(CH_3)_3$), 18.14 (CH_3-5). There are extra peaks from rotamers at 48.85 (CH-4) and 17.11 (CH_3-5) included.

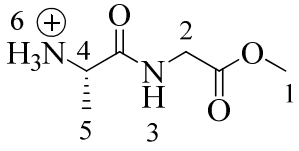
IR (cm^{-1}): 3307 (N-H, br), 2979 (C-H, s), 1754 (O=C, m), 1660 (O=C, s), 1514 (N=O, m), 1366 (N-H, C-N, m), 1246 (C-N, N-H, m).

LRMS (ES$^+$): 283 [(M + Na)$^+$, 100%], 543 [(2M + Na)$^+$, 23%].

HRMS (ES$^+$): $C_{11}H_{20}N_2NaO_5$ (M + Na)$^+$: calculated 283.1264, found 283.1270.

H-*L*-Ala-Gly-OMe 78

Compound **78** was prepared following general procedure section 5.2.2 (1.0 mmol). The title compound was obtained as colorless oil and was used in the next step without further purification (0.10 g, 89%).



^{1}H NMR (300 MHz, d^6-DMSO): δ 8.88 (1NH-3, t, *J* = 6.0 Hz); 8.18 (1NH_3-6, br); 3.88-3.95 (2H-2, 1H-4, m); 3.64 (3H-1, s); 1.36 (3H-5, d, *J* = 8.0 Hz).

^{13}C NMR (75 MHz, d^6-DMSO): δ 171.17 (C), 170.88 (C), 52.82 (OCH_3), 49.64 (CH-4), 41.57 (CH_2-2), 18.08 (CH_3-Ala).

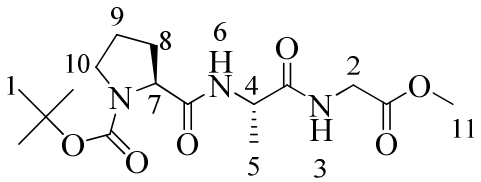
IR (cm^{-1}): 3233 (N-H, br), 2938 (C-H, br), 1737 (C=O, m), 1629 (C=O, s), 1535 (N=O, m), 1366 (N-H, C-N, m), 1261 (C-N, N-H, s).

LRMS (ES^+): 161 [$(M + H)^+$, 100%], 321 [$(2M+H)^+$, 2%].

HRMS (ES^+): $C_6H_{13}N_2O_3$ $(M + H)^+$: calculated 161.0921, found 161.0922.

N-Boc-*L*-Pro-*L*-Ala-Gly-OMe 79

Compound **79** was prepared following general procedure section 5.2.1 (1.1 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 2% MeOH/CH_2Cl_2) and yielded the title compound as colourless oil (1.02 g, 38 %).



^{1}H NMR (400 MHz, d^6-DMSO): δ 8.18 and 8.24 (1NH-3, 2 x br, there are two broad N-H peaks from rotamers); 7.98 (1NH-6, d, *J* = 8.0 Hz); 4.35 (1H-4, m); 4.12 (1H-7, m); 3.84 (2H-2, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.62 (3H-11, s); 3.27 (2H-10, m); 1.71-2.08 (2H-8, 2H-9, m); 1.32 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers); 1.23 (3H-5, d, *J* = 8.0 Hz).

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.60 (C), 173.07 (C), 171.09 (C), 154.22 (C), 79.29 ($C(CH_3)_3$), 60.18 (CH), 52.58 (OCH_3-11), 48.56 (CH), 47.35 (CH_2), 41.35 (CH_2), 31.70 (CH_2), 28.84 ($C(CH_3)_3$), 23.98 (CH_2), 19.19 (CH_3-5). There are extra peaks from rotamers at 172.68 (C), 154.81 (C), 79.65 ($C(CH_3)_3$), 48.69 (CH), 47.55 (CH_2), 30.59 (CH_2), 28.97 ($C(CH_3)_3$), 24.70 (CH_2) and 18.82 (CH_3-5) included.

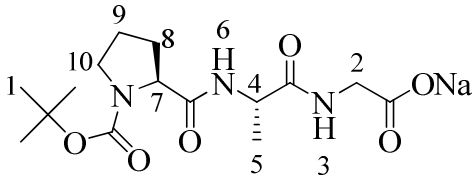
IR (cm^{-1}): 2991 (N-H, br), 2679 (C-H, br), 1671 (O=C, s).

LRMS (ES^+): 358 [$(M + H)^+$, 30%], 737 [$(2M + Na)^+$, 100%].

HRMS (ES^+): $C_{16}H_{27}N_3NaO_6$ $(M + Na)^+$: calculated 380.1792, found 380.1789.

N-Boc-*L*-Pro-*L*-Ala-Gly-ONa 80

Compound **80** was prepared following general procedure section 5.2.4 (1.0 mmol). The desired product was obtained as colorless oil and used in the next step without further purification (0.35 g, 95%).



^{1}H NMR (300 MHz, d^6-DMSO): δ 7.49 (1NH-3, br); 7.12 (1NH-6, br); 4.29 (1H-4, m); 4.11 (1H-7, m); 3.45 (2H-2, d, *J* = 6.0 Hz); 3.32 (2H-10, m); 1.71-2.09 (2H-8, 2H-9, m); 1.31 and 1.37 (9H-1, 2 x s, there are two singlets from rotamers); 1.21 (3H-5, d, *J* = 8.0 Hz).

^{13}C NMR (75 MHz, d^6-DMSO): δ 173.22 (C), 172.80 (C), 172.27 (C), 154.23 (C), 79.31 ($C(CH_3)_3$), 60.16 (CH), 49.08 (CH), 47.44 (CH_2), 44.26 (CH_2), 31.88 (CH_2), 29.02 ($C(CH_3)_3$), 24.22 (CH_2), 19.24 (CH_3-5). There are extra peaks from rotamers at 172.43 (C), 154.60 (C), 47.64 (CH_2), 30.94 (CH_2), 29.17 ($C(CH_3)_3$), 24.89 (CH_2) and 19.12 (CH_3-5).

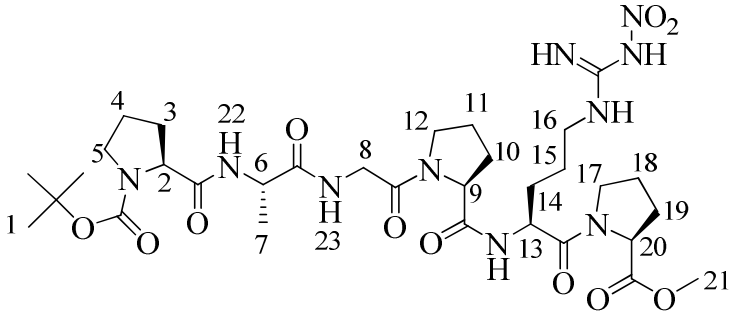
IR (cm^{-1}): 3292 (N-H, br), 2937 (C-H, br), 1727 (C=O, m), 1631 (C=O, s), 1366 (N-H, C-N, s), l262 (C-N, N-H, s).

LRMS (ES$^+$): 388 [(M + Na)$^+$, 100%], 753 [(2M + Na)$^+$, 90%].

HRMS (ES$^+$): $C_{15}H_{25}N_3NaO_6$ (M + Na)$^+$: calculated 366.1636, found 366.1639.

N-Boc-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 81

Compound **81** was prepared following general procedure section 5.2.1 (2.3 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.38 g, 80 %).



m.p.: 62 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.42 (1NH-22, d, *J* = 8.0 Hz); 8.12 (1NH, d, *J* = 8.0 Hz); 8.04 (1NH-23, br); 7.94 (1NH, t, *J* = 4.0 Hz); 7.82 (1NH, br); 4.10-4.52 (1H-2, 1H-9, 1H-13, 1H-20, m); 4.32 (1H-6, m); 3.95 (2H-8, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.60 (3H-21, s); 3.12-3.70 (2H-5, 2H-12, 2H-16, 2H-17, m); 1.55-2.21

(2H-3, 2H-4, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.32 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers); 1.22 (3H-7, d, *J* = 8.0 Hz). There is one of N-H proton missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.18 (C), 173.16 (C), 172.33 (C), 170.75 (C), 167.74 (C), 167.68 (C), 160.24 (C), 154.20 (C), 79.28 (*C*(CH$_3$)$_3$), 60.20 (CH), 59.45 (CH), 55.83 (CH), 54.42 (CH), 52.67 (OCH$_3$-21), 50.87 (CH), 48.88 (CH$_2$), 47.41 (CH$_2$), 46.77 (CH$_2$), 42.16 (CH$_2$), 41.28 (CH$_2$), 31.79 (CH$_2$), 30.75 (CH$_2$), 30.13 (CH$_2$), 29.50 (CH$_2$), 28.93 (C(*C*H$_3$)$_3$), 25.57 (CH$_2$), 25.13 (CH$_2$), 24.11 (CH$_2$), 23.02 (CH$_2$), 19.25 (CH$_3$-7). There are extra peaks from rotamers at 79.53 (*C*(CH$_3$)$_3$), 60.10 (CH), 59.54 (CH), 51.12 (CH), 47.71 (CH$_2$), 46.98 (CH$_2$), 41.87 (CH$_2$), 32.71 (CH$_2$), 29.08 (C(*C*H$_3$)$_3$), 24.78 (CH$_2$) and 19.04 (CH$_3$-7) included.

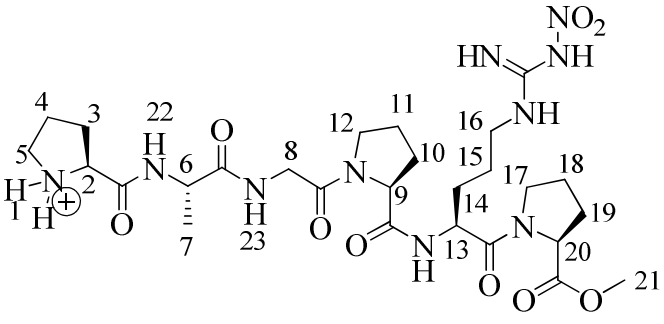
IR (cm^{-1}): 3294 (N-H, br), 2970 (C-H, br), 1740 (O=C, m), 1635 (O=C, s), 1541 (N=O, m), 1365 (N-H, C-N, s), 1231 (C-N, N-H, s).

LRMS (ES^{+}): 399 [(M+2Na)$^{2+}$, 29%], 775 [(M + Na)$^{+}$, 100%], 1528 [(2M+Na)$^{+}$, 9%].

HRMS (ES^{+}): $C_{32}H_{52}N_{19}NaO_{11}$ (M + Na)$^{+}$: calculated 775.3709, found 775.3721.

H-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 82

Compound **82** was prepared following general procedure section 5.2.3 (0.14 mmol). The title compound was obtained as colorless oil and was used in the next step without further purification (0.09 g, 97%).



m.p.: 98 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 9.84 (1NH, br); 8.72 (1NH-22, d, *J* = 8.0 Hz); 8.42-8.50 (2NH, br); 8.15 (1NH, d, *J* = 8.0 Hz); 8.09-8.16 (2NH, br); 8.04 (1NH-23, br); 3.82-4.52 (1H-2, 1H-6, 1H-9, 1H-13, 1H-20, m); 4.00 (2H-8, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.61 (3H-21, s); 3.12-3.68 (2H-5, 2H-12, 2H-16, 2H-17, m); 1.49-

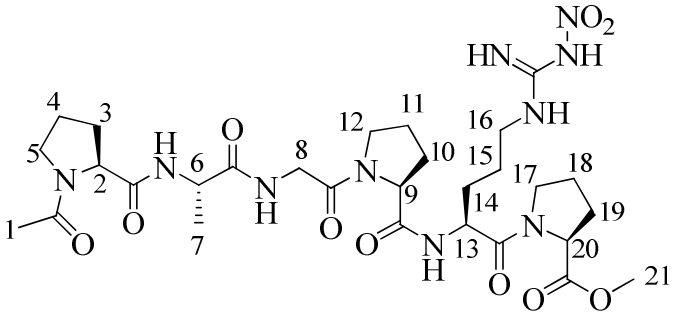
2.33 (2H-3, 2H-4, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.27 (3H-7, d, *J* = 8.0 Hz).

LRMS (ES$^+$): 653 [(M+H)$^+$, 44%], 675 [(M + Na)$^+$, 53%].

HRMS (ES$^+$): $C_{27}H_{45}N_{19}O_9$ (M + H)$^+$: calculated 653.3365, found 653.3357.

N-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 83

Compound **83** was prepared following general procedure section 5.2.5 (0.14 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2). The product was precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.05 g, 58%).



m.p.: 66 ˚C

^{1}H NMR (400 MHz, $CDCl_3$): δ 11.93 (1NH, d, *J* = 8.0 Hz); 9.47 (1NH, br); 7.51 (1NH, d, *J* = 8.0 Hz); 6.92 (1NH, d, *J* = 4.0 Hz); 3.88-4.57 (1H-2, 1H-6, 1H-9, 1H-13, 1H-20, m); 4.02 (2H-8, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.73 (3H-21, s); 3.08-3.56 (2H-5, 2H-12, 2H-16, 2H-17, m); 2.14 and 2.18 (3H-1, s, there are two singlets from rotamers); 1.70-2.33 (2H-3, 2H-4, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.41 and 1.42 (3H-7, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers). There are 2 x N-H protons missing from ^{1}H spectrum.

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.02 (C), 172.37 (C), 172.29 (C), 172.10 (C), 171.55 (C), 171.40 (C), 168.72 (C), 154.93 (C), 60.68 (CH), 52.62 (CH), 52.45 (OCH_3-21), 52.06 (CH), 48.54 (CH), 48.21 (CH), 46.97 (CH_2), 45.86 (CH_2), 42.22 (CH_2), 41.20 (CH_2), 41.12 (CH_2), 38.23 (CH_2), 29.06 (CH_2), 28.56 (CH_2), 25.38 (CH_2), 25.43 (CH_2), 25.33 (CH_2), 25.11 (CH_2), 24.78 (CH_2), 22.50 (CH_3-Ac), 17.79 (CH_3-7). There are extra peaks from rotamers at 155.02 (C), 60.62 (CH), 25.17 (CH_2) and 25.07 (CH_2) included.

IR (cm^{-1}): 3272 (N-H, br), 2952 (C-H, br), 1622 (C=O, s), 1539 (N=O, m), 1270 (C-N, N-H, m).

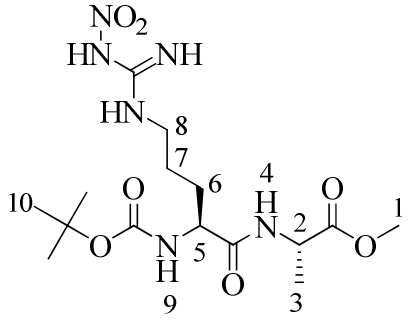
LRMS (ES$^+$): 239 [(M+Na+2H)$^{3+}$, 25%], 370 [(M+2Na)$^{2+}$, 4%], 717 [(M+Na)$^+$, 100%], 1411 [(2M+Na)$^+$, 6%]

HRMS (ES$^+$): $C_{29}H_{46}N_{10}NaO_{10}$ (M + Na)$^+$: calculated 717.3291, found 717.3295.

c) Synthesis of Ac-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Ala-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 90.

***N*-Boc-*L*-Arg(NO$_2$)-*L*-Ala-OMe 84**

Compound **84** was prepared following general procedure section 5.2.1 (6.3 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10% MeOH) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.78 g, 70 %).



m.p.: 65 ºC

^{1}H NMR (300 MHz, d^6-DMSO): δ 8.23 (1NH-4, d, *J* = 8.0 Hz); 6.82 (1NH-9, d, *J* = 8.0 Hz); 4.26 (1H-2, apparent p, *J* = 8.0 Hz); 3.93 (1H-5, m); 3.60 (3H-1, s); 3.14 (2H-8, m); 1.48-1.62 (2H-6, 2H-7, m); 1.37 (9H-10, s); 1.28 (3H-3, d, *J* = 8.0 Hz). There are 3 x N-H protons from guanidine side chain missing from this spectrum.

^{13}C NMR (100 MHz, d^6-DMSO): δ 177.95 (C), 173.87 (C), 172.78 (C), 156.15 (C), 78.97 (*C*(CH$_3$)$_3$), 54.40 (CH), 52.74 (OCH$_3$-1), 48.38 (CH), 41.24 (CH$_2$), 30.15 (CH$_2$), 29.11 (C(*C*H$_3$)$_3$), 17.64 (CH$_3$-3). There is one CH$_2$ peak missing from ^{13}C-spectrum because overlapping of CH$_2$ peaks.

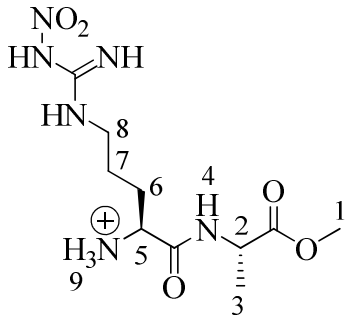
IR (cm^{-1}): 3302 (N-H, br), 2971 (C-H, br), 1740 (C=O, m), 1652 (C=O, m), 1597 (N=O, m), 1366 (N-H, C-N, s), 1251 (C-N, N-H, s).

LRMS (ES$^+$): 427 [(M + Na)$^+$, 100%], 831 [(2M+Na)$^+$, 22%].

HRMS (ES$^+$): $C_{15}H_{28}N_6NaO_7$ (M + Na)$^+$: calculated 427.1912, found 427.1910.

H-*L*-Arg(NO_2)-*L*-Ala-OMe 85

Compound **85** was prepared following general procedure section 5.2.2 (1.0 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (1.16 g, 87%).



m.p.: 90 °C

^{1}H NMR (300 MHz, d^6-DMSO): δ 8.90 (1NH-4, d, *J* = 8.0 Hz); 8.62 (1NH, br); 8.21 (1NH_3-9, br); 7.97 (1NH, br); 4.36 (1H-2, apparent p, *J* = 8.0 Hz); 3.79 (1H-5, m); 3.64 (3H-1, s); 3.18 (2H-8, m); 1.58-1.73 (2H-6, 2H-7, m); 1.33 (3H-3, d, *J* = 8.0 Hz).

There is 1 x N-H proton from guanidine side chain missing from ^{1}H-spectrum.

^{13}C NMR (75 MHz, d^6-DMSO): δ 173.34 (C), 169.32 (C), 160.27 (C), 52.99 (CH), 52.67 (OCH_3-1), 48.65 (CH), 40.89 (CH_2), 29.33 (CH_2), 17.68 (CH_3-3). There is one CH_2 peak missing from ^{13}C-spectrum because the overlapping of CH_2 peaks.

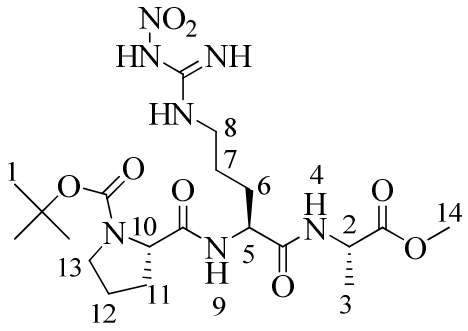
IR (cm^{-1}): 2970 (C-H, br), 1739 (C=O, s), 1684 (C=O, m), 1558 (N=O, m), 1365 (N-H, C-N, s), 1229 (C-N, N-H, s).

LRMS (ES$^+$): 305 [(M + H)$^+$, 100%], 609 [(2M+H)$^+$, 9%].

HRMS (ES$^+$): $C_{10}H_{21}N_6O_5$ (M + H)$^+$: calculated 305.1568, found 305.1573.

***N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe 86**

Compound **86** was prepared following general procedure section 5.2.1 (6.5 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10% MeOH) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (2.12 g, 65 %).



m.p.: 71 °C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.55 (1NH, br); 8.25 and 8.39 (1NH-9, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers); 7.86 (1NH-4, d, *J* = 8.0 Hz); 4.29 (1H-10, m); 4.25 (1H-2, apparent p, *J* = 8.0 Hz); 4.13 (1H-5, m); 3.61 (3H-14, s); 3.34 (2H-8, m); 3.15 (2H-13, m); 2.08 (2H-6, m); 1.74 (2H-7, m); 1.70 (2H-11, m); 1.50 (2H-12, m); 1.31 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers); 1.28 (3H-3, d, *J* = 8.0 Hz). There are 2 x N-H protons of guanidine side chain missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.77 (C), 173.26 (C), 172.24 (C), 160.24 (C), 154.27 (C), 79.37 (*C*$(CH_3)_3$), 60.31 (CH), 52.77 (CH), 52.57 (OCH_3-1), 48.47 (CH), 47.45 (CH_2), 41.22 (CH_2), 31.92 (CH_2), 30.49 (CH_2), 30.26 (CH_2), 28.91 ($C(CH_3)_3$), 24.01 (CH_2), 17.75 (CH_3-3). There are extra peaks from rotamers at 79.63 (*C*$(CH_3)_3$), 60.20 (CH), 47.66 (CH_2), 30.68 (CH_2), 29.04 ($C(CH_3)_3$) and 24.86 (CH_2) included.

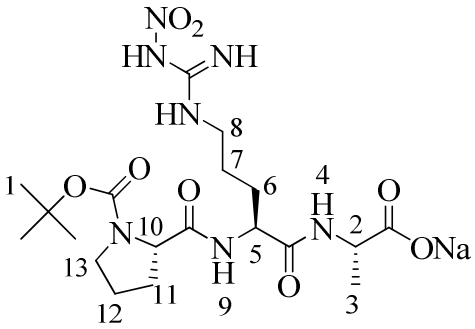
IR (cm^{-1}): 3290 (N-H, br), 2971 (C-H, br), 1741 (C=O, m), 1654 (C=O, s), 1535 (N=O, m), 1366 (N-H, C-N, m), 1257 (C-N, N-H, s).

LRMS (ES^{+}): 524 [(M + Na)$^{+}$, 100%], 1025 [(2M+Na)$^{+}$, 8%].

HRMS (ES^{+}): $C_{20}H_{35}N_7NaO_8$ (M + Na)$^{+}$: calculated 524.2439, found 524.2440.

N-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-ONa 87

Compound **87** was prepared following general procedure section 5.2.4 (0.6 mmol). The title product was obtained as a white solid and was use in the next step without further purification (0.22 g, 74%).



m.p.: 101 °C.

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 7.63-8.48 (3NH, br); 4.16-4.17 (1H-10, 1H-2, m); 3.75 (1H-5, m); 3.17-3.30 (2H-8, 2H-13, m); 2.14 (2H-6, m); 1.73 (2H-7, m); 1.50-1.76 (2H-12, 2H-11, m); 1.30 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers); 1.15 (3H-3, d, J = 8.0 Hz). There are 2 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.23 (C), 171.65 (C), 170.76 (C), 160.33 (C), 154.28 (C), 79.29 (C(CH$_3$)$_3$), 60.23 (CH), 50.81 (CH), 49.52 (CH), 47.60 (CH$_2$), 47.44 (CH$_2$), 39.02 (CH$_2$), 29.99 (CH$_2$), 28.93 (C(CH$_3$)$_3$), 25.06 (CH$_2$), 23.99 (CH$_2$), 20.01 (CH$_3$-Ala). There are extra peaks from rotamers at 153.49 (C) and 29.07 (C(CH$_3$)$_3$) included.

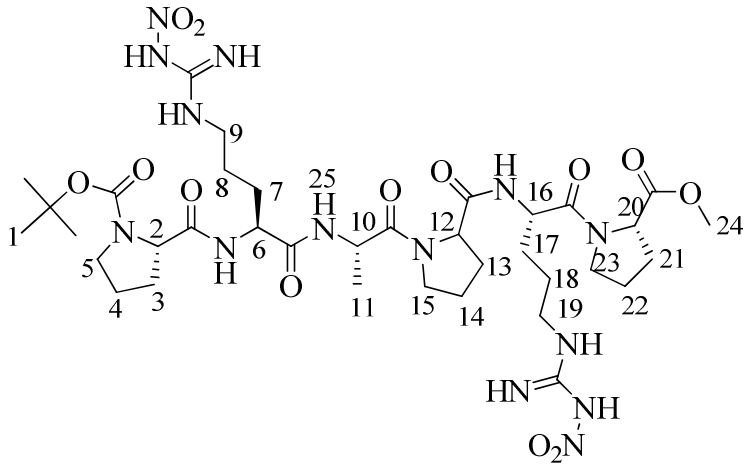
IR (cm^{-1}): 3299 (N-H, br), 2978 (C-H, br), 1629 (C=O, s), 1529 (N=O, m), 1367 (N-H, C-N, m), 1258 (C-N, N-H, s).

LRMS (ES^{+}): 532 [(M + Na)$^{+}$, 70%], 1041 [(2M+Na)$^{+}$, 10%].

HRMS (ES^{+}): $C_{19}H_{32}N_7Na_2O_8$ (M + Na)$^{+}$: calculated 532.2102, found 532.2113.

N-Boc-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Ala-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Pro-OMe 88

Compound **88** was prepared following general procedure section 5.2.1 (1.0 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.54 g, 60 %).



m.p.: 78 °C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.52 (1NH, br); 8.31 (1NH, d, *J* = 8.0 Hz); 8.22 (1NH, d, *J* = 8.0 Hz); 8.04 (1NH-25, d, *J* = 8.0 Hz); 7.69-8.02 (3NH, br); 7.53 (1NH, t, *J* = 6.0 Hz); 7.40 (1NH, br); 4.12-4.75 (1H-2, 1H-6, 1H-12, 1H-16, 1H-20, m); 4.30 (1H-10, m); 3.60 and 3.58 (3H-24, s, there are two singlet from rotamers); 3.16-3.75 (2H-5, 2H-9, 2H-15, 2H-19, 2H-23, m); 1.50-2.21 (2H-3, 2H-4, 2H-7, 2H-8, 2H-13, 2H-14, 2H-17, 2H-18, 2H-21, 2H-22, m); 1.30 and 1.37 (9H-1, 2 x s, there are two singlets from rotamers); 1.17 and 1.16 (3H-11, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.56 (C), 173.39 (C), 173.22 (C), 172.25 (C), 171.81 (C), 171.47 (C), 171.11 (C), 170.79 (C), 160.30 (C), 79.43 (*C*(CH$_3$)$_3$), 60.38 (CH), 60.15 (CH), 59.48 (CH), 55.85 (CH), 52.75 (OCH$_3$-24), 50.93 (CH), 50.74 (CH), 47.69 (CH$_2$), 47.55 (CH$_2$), 47.45 (CH$_2$), 41.32 (CH$_2$), 41.23 (CH$_2$), 41.16 (CH$_2$), 31.99 (CH$_2$), 30.73 (CH$_2$), 29.44 (CH$_2$), 29.54 (CH$_2$), 28.95 (C(*C*H$_3$)$_3$), 25.61 (CH$_2$), 25.46 (CH$_2$), 25.09 (CH$_2$), 24.90 (CH$_2$), 24.04 (CH$_2$), 18.13 (CH$_3$-Ala). There are extra peaks from rotamers at 173.11 (C), 172.30 (C), 171.87 (C), 170.70 (C), 79.53 (*C*(CH$_3$)$_3$), 60.55 (CH), 59.39 (CH), 52.68 (OCH$_3$), 51.19 (CH), 47.19 (CH$_2$), 47.02 (CH$_2$), 41.10 (CH$_2$), 32.73(CH$_2$), 30.36(CH$_2$), 30.15 (CH$_2$), 29.25 (CH$_2$), 23.00 (CH$_2$) and 18.36 (CH$_3$-11) included.

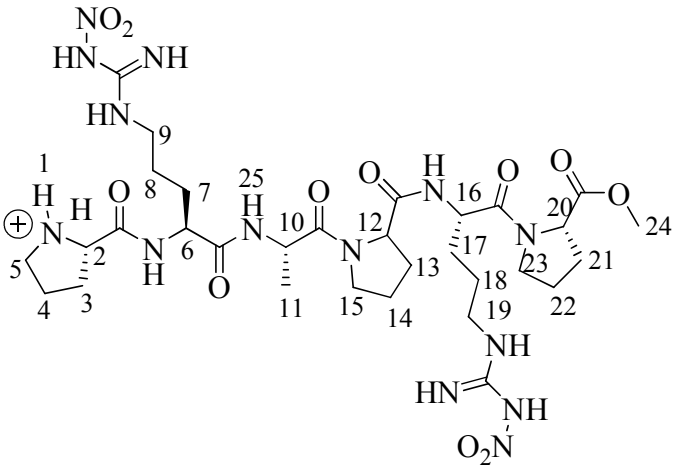
IR (cm^{-1}): 3293 (N-H, br), 2978 (C-H, br), 1622 (C=O, s), 1531 (N=O, m), 1254 (C-N, N-H, s).

LRMS (ES^{+}): 471 [(M+2Na)$^{2+}$, 2%], 919 ([(M+Na)$^{+}$, 7%]).

HRMS (ES^{+}): $C_{36}H_{60}N_{14}NaO_{13}$ (M + Na)$^{+}$: calculated 919.4356, found 919.4346.

H-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 89

Compound **89** was prepared following general procedure section 5.2.3 (0.11 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.09 g, 92%).



m.p.: 98 °C

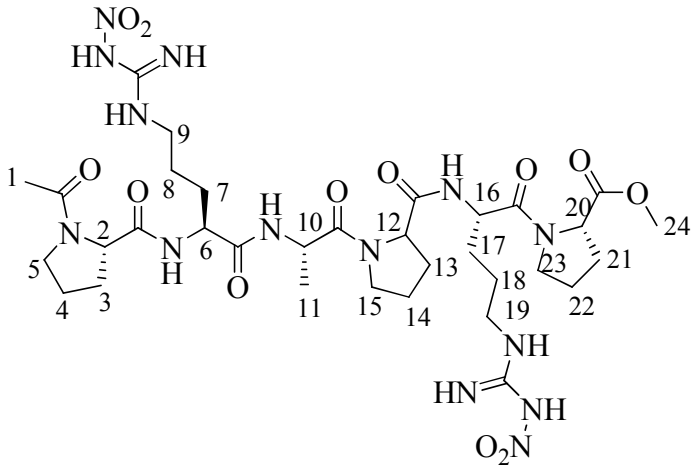
^{1}H NMR (400 MHz, d^6-DMSO): δ 8.63 (1NH, t, *J* = 8.0 Hz); 8.54 (1NH, br); 8.20 (1NH, d, *J* = 8.0 Hz); 8.14 (1NH, br); 8.02 (1NH-25, d, *J* = 8.0 Hz); 7.92 (4NH, br); 4.20-4.60 (1H-2, 1H-6, 1H-12, 1H-16, 1H-20, m); 4.33 (1H-10, m); 3.61 (3H-24, s); 3.17-3.65 (2H-5, 2H-9, 2H-15, 2H-19, 2H-23, m); 1.53-2.34 (2H-3, 2H-4, 2H-7, 2H-8, 2H-13, 2H-14, 2H-17, 2H-18, 2H-21, 2H-22, m); 1.19 and 1.09 (3H-11, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers).

LRMS (ES$^+$): 410 [$(M+H+Na)^{2+}$, 3%], 797 [$(M+H)^+$, 58%], 819 [$(M+Na)^+$, 22%].

HRMS (ES$^+$): $C_{31}H_{53}N_{14}O_{11}$ $(M + H)^+$: calculated 797.4013, found 797.4021.

***N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe 90**

Compound **90** was prepared following general procedure section 5.2.5 (0.1 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2). The product was precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.07 g, 77%).



m.p.: 56 °C

^{1}H NMR (400 MHz, $CDCl_3$): δ 11.81-11.97 (1NH, br); 9.40 (1NH, d, *J* = 8.0 Hz); 6.95-8.14 (7NH, br); 4.37-4.71 (1H-2, 1H-6, 1H-10, 1H-12, 1H-16, 1H-20, m); 3.67 and 3.62 (3H-24, s, there are two singlets from rotamers); 3.03-3.93 (2H-5, 2H-9, 2H-15, 2H-19, 2H-23, m); 2.05 (3H-1, s); 1.61-2.36 ((2H-3, 2H-4, 2H-7, 2H-8, 2H-13, 2H-14, 2H-17, 2H-18, 2H-21, 2H-22, m); 1.33 and 1.34 (3H-11, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, $CDCl_3$): δ 172.78 (C), 172.47 (C), 172.38 (C), 172.23 (C), 172.13 (C), 171.36 (C), 171.20 (C), 170.76 (C), 154.89 (C), 61.05 (CH), 60.51 (CH), 60.27 (CH), 58.86 (CH), 52.38 (OCH_3-24), 50.95 (CH), 50.41 (CH), 48.52 (CH_2), 48.14 (CH_2), 47.47 (CH_2), 46.88 (CH_2), 45.86 (CH_2), 41.47 (CH_2), 40.78 (CH_2), 41.38 (CH_2), 29.88 (CH_2), 29.22 (CH_2), 28.85 (CH_2), 27.88 (CH_2), 25.30 (CH_3-1), 25.03 (CH_2), 24.96 (CH_2), 24.14 (CH_2), 22.43 (CH_3-11). There are extra peaks from rotamers at 52.44 and 52.30 (OCH_3-24), 48.42 (CH_2), 28.97 (CH_2), 25.11 (CH_3-1), 24.76 (CH_2), 22.57 (CH_3-11).

IR (cm^{-1}): 3272 (N-H, br), 2952 (C-H, br), 1717 (C=O, m), 1622 (C=O, s), 1559 (N=O, m), 1373 (N-H, C-N, s), 1272 (C-N, N-H, s).

LRMS (ES^{+}): 442 [$(M+2Na)^{2+}$, 19%], 861 [$(M+Na)^{+}$, 100%].

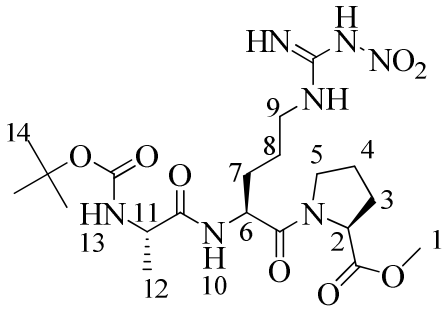
HRMS (ES^{+}): $C_{33}H_{54}N_{14}NaO_{12}$ $(M + Na)^{+}$: calculated 861.3938, found 861.3932.

d) Synthesis of Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe 95.

N-Boc-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe 91

Compound **91** was prepared following general procedure section 5.2.1 (5.9 mmol). Crude residue was purified by column chromatography (30% EtOAc/ 60% Petroleum ether/ 10%

MeOH) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.71 g, 58 %).



m.p.: 58 ˚C

^{1}H NMR (300 MHz, d^6-DMSO): δ 7.89-7.91 (1NH-10, d, *J* = 8.0 Hz and 1NH, br); 6.92 (1NH-13, d, *J* = 8.0 Hz); 4.52 (1H-6, m); 4.28 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.02 (1H-11, m); 3.63 (2H-5, m); 3.60 (3H-1, s); 3.28 (2H-9, m); 1.53-2.20 (2H-7, 2H-8, 2H-3, 2H-4, m); 1.36 (9H-14, s); 1.14 (3H-12, d, *J* = 8.0 Hz). There are 2 x N-H protons from guanidine side chain missing from ^{1}H-spectrum.

^{13}C NMR (75 MHz, d^6-DMSO): δ 173.59 (C), 173.15 (C), 170.79 (C), 160.30 (C), 155.95 (C), 79.06 (*C*(CH$_3$)$_3$), 60.70 (CH), 59.45 (CH), 52.73 (OCH$_3$-1), 50.63 (CH), 47.47 (CH$_2$), 41.30 (CH$_2$), 29.50 (CH$_2$), 29.42 (CH$_2$), 29.09 (C(*C*H$_3$)$_3$), 25.57 (CH$_2$), 19.05 (CH$_3$-12). There is one CH$_2$ peaak missing because overlapping of CH$_2$ peaks from ^{13}C spectrum.

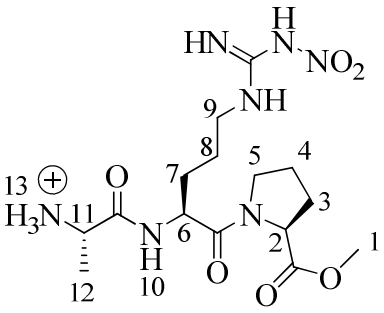
IR (cm^{-1}): 3305 (N-H, br), 2979 (C-H, s), 1626 (O=C, s), 1522 (N=O, m), 1366 (N-H, C-N, m), 1250 (C-N, N-H, s).

LRMS (ES$^+$): 524 [(M + Na)$^+$, 100%], 1025 [(2M + Na)$^+$, 62%], 1527 [(3M+Na)$^+$, 15%]

HRMS (ES$^+$): $C_{20}H_{35}N_7NaO_8$ (M + Na)$^+$: calculated 524.2439, found 524.2433.

H-*L*-Ala-*L*-Arg(NO$_2$)-*L*-Pro-OMe 92

Compound **92** was prepared following general procedure section 5.2.2 (1.0 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.41 g, 79%).



m.p.: 73 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.63 (1NH-10, d, *J* = 8.0 Hz); 8.11 (3NH-13, br); 7.97 (1NH, br); 4.53 (1H-6, m); 4.31 (1H-2, dd, *J* = 5.0, 9.0 Hz); 3.88 (1H-11, m); 3.71 (2H-5, m); 3.61 and 3.66 (3H-1, 2 x s, there are two singlets from rotamers); 3.18 (2H-9, m); 2.18 (2H-3, apparent dt, *J* = 8.0, 8.0, 12.0 Hz); 1.94 (2H-4, apparent tt, *J* = 4.0, 4.0, 12.0, 12.0 Hz); 1.73 (2H-7, apparent t, *J* = 4.0 Hz); 1.58 (2H-8, m); 1.31 (3H-12, d, *J* = 8.0 Hz). There are 2 x N-H protons from guanidine side chain missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.08 (C), 170.46 (C), 170.31 (C), 159.33 (C), 59.51 (CH), 52.74 (OCH_3-1), 51.31 (CH), 48.91 (CH), 47.56 (CH_2), 47.08 (CH_2), 41.23 (CH_2), 29.52 (CH_2), 29.13 (CH_2), 25.62 (CH_2), 18.08 (CH_3-12). There are extra peaks from rotamers at 170.73 (C), 59.75 (CH), 53.36 (OCH_3-1), 25.29 (CH_2), 18.22 (CH_3-12) and HOBt at 121.52 (CH), 118.61 (CH), 115.68 (CH) and 112.76 (CH) included.

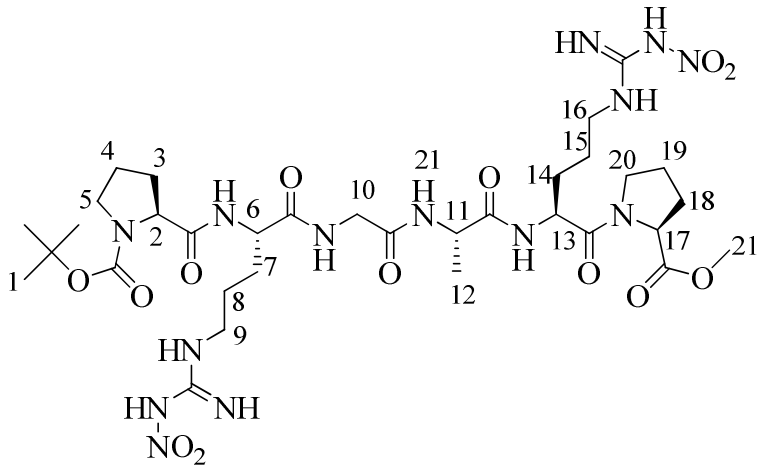
IR (cm^{-1}): 3281 (N-H, br), 2359 (C-H, br), 1747 (C=O, m), 1660 (C=O, s), 1539 (N=O, m), 1261 (C-N, N-H, s).

LRMS (ES^{+}): 402 [$(M + H)^{+}$, 100%], 803 [(2M+H)+, 5%],

HRMS (ES^{+}): $C_{15}H_{28}N_7O_6$ $(M + H)^{+}$: calculated 401.2096, found 401.2101.

N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe 93

Compound **93** was prepared following general procedure section 5.2.1 (1.8 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.96 g, 62 %).



m.p.: 83.1-83.9 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.51 (2NH, br); 8.17 (1NH, d, *J* = 8.0 Hz); 8.09 (1NH, d, *J* = 8.0 Hz); 7.99 (1NH, t, *J* = 6.0 Hz); 7.89-7.94 (1NH-21, d, *J* = 8.0 Hz, 4NH, br); 4.30 (1H-11, apparent p, *J* = 8.0 Hz); 4.12-4.48 (1H-2, 1H-6, 2H-10, 1H-13, 1H-17, m); 3.60 (3H-21, s); 3.11-3.71 (2H-5, 2H-9, 2H16, 2H-20, m); 1.54-2.19 (2H-3, 2H-4, 2H-7, 2H-8, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.30 and 1.37 (9H-1, 2 x s, there are two singlets from rotamers), 1.25 and 1.17 (3H-12, d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.56 (C), 173.25 (C), 173.16 (C), 172.91 (C), 172.69 (C), 170.77 (C), 169.15 (C), 160.27 (C), 154.25 (C), 79.39 (*C*(CH$_3$)$_3$), 59.74 (CH), 58.94 (CH), 53.89 (CH), 52.81 (CH), 52.19 (OCH$_3$-21), 50.39 (CH), 48.34 (CH$_2$), 46.96 (CH$_2$), 42.33 (CH$_2$), 40.70 (CH$_2$), 31.99 (CH$_2$), 31.40 (CH$_2$), 30.14 (CH$_2$), 29.65 (CH$_2$), 29.02 (CH$_2$), 28.68 (CH$_2$), 28.44 (C(*C*H$_3$)$_3$), 25.09 (CH$_2$), 24.38 (CH$_2$), 23.53 (CH$_2$), 18.65 (CH$_3$-12). There are extra peaks from rotamers at 79.69 (*C*(CH$_3$)$_3$), 47.14 (CH$_2$), 42.40 (CH$_2$) and 28.56 (C(*C*H$_3$)$_3$) included.

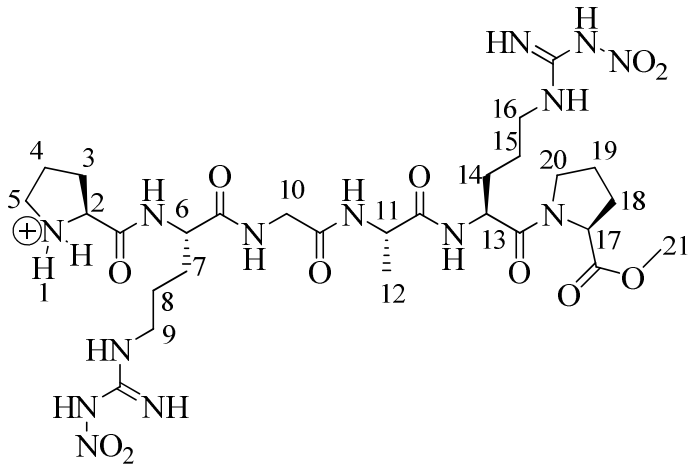
IR (cm^{-1}): 3294 (N-H, br), 2970 (C-H, br), 1739 (C=O, s), 1634 (C=O, s), 1539 (N=O, s), 1366 (N-H, C-N, s), 1230 (C-N, N-H, s)..

LRMS (ES^{+}): 879 [(M + Na)$^{+}$, 7%].

HRMS (ES^{+}): $C_{33}H_{56}N_{14}NaO_{13}$ (M + Na)$^{+}$: calculated 879.4043, found 879.4052.

H-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Ala-*L*-Arg(NO$_2$)-*L*-Pro-OMe 94

Compound **94** was prepared following general procedure section 5.2.3 (0.14 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.11 g, 97%).



m.p.: 97 ˚C

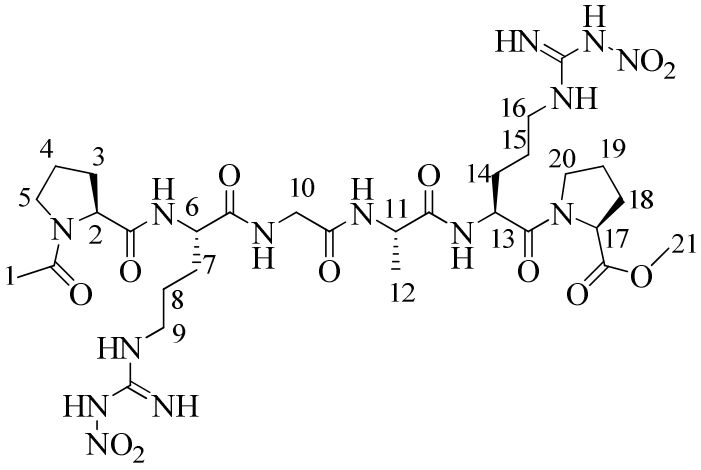
^{1}H NMR (400 MHz, d^6-DMSO): δ 9.38 (1NH, br); 8.72 (1NH, d, *J* = 8.0 Hz); 8.50-8.52 (3NH, br); 8.27 (1NH, t, *J* = 6.0 Hz); 8.15 (1NH, d, *J* = 8.0 Hz), 7.97 (1NH, d, *J* = 8.0 Hz); 4.21-4.49 (1H-2, 1H-6, 2H-10, 1H-11, 1H-13, 1H-17, m); 3.60 (3H-21, s); 3.13-3.75 (2H-5, 2H-9, 2H16, 2H-20, m); 1.54-2.27 (2H-3, 2H-4, 2H-7, 2H-8, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.25 and 1.17 (3H-12, d x 2, there are two doublets from rotamers, *J* = 8.0 Hz). There are 4 x N-H protons missing from ^{1}H-spectrum.

LRMS (ES$^+$): 757 [(M + H)$^+$, 23%], 779 [(M+Na)$^+$, 8%].

HRMS (ES$^+$): $C_{28}H_{49}N_{14}O_{11}$ (M + H)$^+$: calculated 757.3700, found 757.3695.

N-Ac-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Ala-*L*-Arg(NO$_2$)-*L*-Pro-OMe 95

Compound **95** was prepared following general procedure section 5.2.5 (0.14 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2). The product was precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.07 g, 65%).



m.p.: 97 ˚C

^{1}H NMR (400 MHz, $CDCl_3$): δ 11.92 (1NH, br); 9.51 (1NH, br); 7.50-7.93 (7 x NH, br); 7.37 (1NH, d, *J* = 8.0 Hz); 3.99-4.73 (1H-2, 1H-6, 2H-10, 1H-11, 1H-13, 1H-17, m); 3.72 (3H-21, s); 3.11-3.82 (2H-5, 2H-9, 2H16, 2H-20, m); 2.18 (3H-1, s); 1.73-2.25 (2H-3, 2H-4, 2H-7, 2H-8, 2H-14, 2H-15, 2H-18, 2H-19, m), 1.40 and 1.41 (3H-12, d, *J* = 8.0 Hz, there are two doublets from rotamers). There are 4 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.08 (C), 172.25 (C), 172.18 (C), 171.68 (C), 171.41 (C), 170.80 (C), 170.19 (C), 168.55 (C), 154.74 (C), 60.51 (CH), 60.38 (CH), 58.95 (CH), 52.27 (OCH_3-21), 50.27 (CH), 48.31 (CH), 48.39 (CH_2), 47.06 (CH_2), 46.71 (CH_2), 46.69 (CH_2), 42.21 (CH_2), 41.44 (CH_2), 28.93 (CH_2), 28.88 (CH_2), 28.62 (CH_2), 28.44 (CH_2), 24.97 (CH_2), 24.76 (CH_2), 24.24 (CH_2), 22.56 (CH_3-1), 17.62 (CH_3-12).

IR (cm^{-1}): 3271 (N-H, br), 2974 (C-H, br), 1738 (C=O, m), 1622 (C=O, s), 1539 (N=O, m), 1372 (N-H, C-N, m), 1269 (C-N, N-H, s).

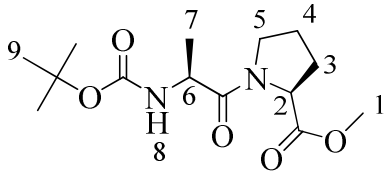
LRMS (ES$^+$): 422 [$(M+2Na)^{2+}$, 29%], 821 [$(M+Na)^+$, 100%], 1619 [$(2M+Na)^+$, 4%].

HRMS (ES$^+$): $C_{30}H_{50}N_{14}NaO_{12}$ $(M + Na)^+$: calculated 821.3625, found 821.3645.

e) Synthesis of Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe 102.

N-Boc-*L*-Ala-*L*-Pro-OMe 96

Compound **96** was prepared following general procedure section 5.2.1 (10.6 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 2% MeOH/CH_2Cl_2) and yielded the title compound as colourless oil (1.49 g, 47 % yield).



^{1}H NMR (400 MHz, d^6-DMSO): δ 6.93 (1NH-8, d, *J* = 8.0 Hz); 4.32 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.24 (1H-6, apparent p, *J* = 8.0 Hz); 3.60 and 3.65 (3H-1, s, there are two singlets from rotamers); 3.63 (1H-5, m); 3.53 (1H-5', m); 2.17 (1H-3, m); 1.93 (2H-4, apparent p, *J* = 8.0 Hz); 1.80 (1H-3', m); 1.36 (9H-9, s); 1.14 and 1.18 (3H-7, d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.25 (C), 172.15 (C), 155.93 (C), 78.85 ($C(CH_3)_3$), 59.34 (CH), 52.63 (OCH_3-1), 48.35 (CH_2), 47.17 (CH), 29.12 ($C(CH_3)_3$), 29.36 (CH_2),

25.59 (CH_2), 17.51 (CH_3-7). There are extra peaks from rotamers at 53.10 (OCH_3-1), 47.09 (CH), 28.88 ($C(CH_3)_3$) and 17.95 (CH_3-7) included.

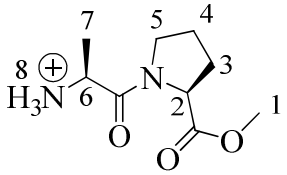
IR (cm^{-1}): 3324 (N-H, br), 2977 (C-H, br), 1744 (C=O, m), 1503 (N=O, m), 1365 (N-H, C-N, s), 1246 (C-N, N-H, m).

LRMS (ES^+): 323 [$(M + Na)^+$, 56%], 623 [$(2M + Na)^+$, 100%].

HRMS (ES^+): $C_{14}H_{24}N_2NaO_5$ $(M + Na)^+$: calculated 323.1577, found 323.1580.

H-*L*-Ala-*L*-Pro-OMe 97

Compound **97** was prepared following general procedure section 5.2.2 (0.7 mmol). The title compound was obtained as colorless oil and was used in the next step without further purification (0.19 g, 86%).



^{1}H NMR (300 MHz, d^6-DMSO): δ 8.20 (3NH-8, br); 4.38 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.23 (1H-6, m); 3.70 (1H-5, m); 3.62 (3H-1, s); 3.48 (1H-5', m); 2.23 (1H-3, m); 1.92 (2H-4, m); 1.85 (1H-3', m); 1.34 (3H-7, d, *J* = 8.0 Hz).

^{13}C NMR (75 MHz, d^6-DMSO): δ 172.81 (C), 169.12 (C), 59.60 (CH), 52.94 (OCH_3), 48.02 (CH), 47.43 (CH_2), 29.48 (CH_2), 25.75 (CH_2), 16.31 (CH_3-Ala).

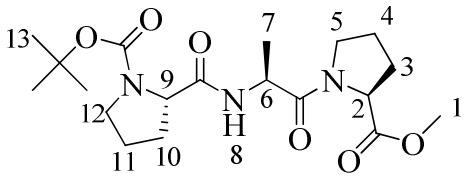
IR (cm^{-1}): 3232 (N-H, br), 2939 (C-H, br), 1736 (C=O, m), 1629 (C=O, s), 1535 (N=O, s), 1366 (N-H, C-N, s), 1262 (C-N, N-H, s).

LRMS (ES^+): 201 [$(M + H)^+$, 100%], 401 [$(2M + H)^+$, 25%].

HRMS (ES^+): $C_9H_{17}N_2O_3$ $(M + Na)^+$: calculated 201.1234, found 201.1235.

N-Boc-*L*-Pro-*L*-Ala-*L*-Pro-OMe 98

Compound **98** was prepared following general procedure section 5.2.1 (7.5 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 2% MeOH/CH_2Cl_2) and yielded the title compound as colourless oil (1.21 g, 43% yield).



^{1}H NMR (300 MHz, d^{6}-DMSO): δ 8.10 (1NH-8, d, *J* = 8.0 Hz); 4.52 (1H-6, apparent p, *J* = 8.0 Hz); 4.30 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.10 (1H-9, m); 3.60 and 3.63 (3H-1, 2 x s, there are two singlets from rotamers); 3.56 (2H-12, m); 3.32 (2H-5, m); 1.72-2.20 (2H-3, 2H-4, 2H-10, 2H-11, m); 1.32, 1.34, 1.38 and 1.39 (9H-13, 4 x s, there are four singlets from rotamers); 1.20 (3H-7, d, *J* = 8.0 Hz).

^{13}C NMR (75 MHz, d^{6}-DMSO): δ 175.30 (C), 173.19 (C), 171.43 (C), 154.20 (C), 79.41 (*C*(CH$_3$)$_3$), 60.03 (CH), 59.36 (CH), 52.67 (OCH$_3$-1), 47.39 (CH), 47.30 (CH$_2$), 46.83 (CH$_2$), 31.79 (CH$_2$), 29.42 (CH$_2$), 28.92 (C(*C*H$_3$)$_3$), 25.57 (CH$_2$), 24.04 (CH$_2$), 17.88 (CH$_3$-7). There are extra peaks from rotamers at 174.92 (C), 172.91 (C), 172.53 (C), 154.51 (C), 154.07 (C), 79.25 (*C*(CH$_3$)$_3$), 53.17 (OCH$_3$-1), 47.58 (CH$_2$), 47.00 (CH$_2$), 31.22 (CH$_2$), 30.71 (CH$_2$), 29.04 and 28.87 (C(*C*H$_3$)$_3$), 24.73 (CH$_2$), 17.57 (CH$_3$-7) included.

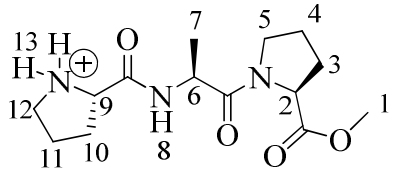
IR (cm^{-1}): 3287 (N-H, br), 1733 (C=O, m), 1634 (C=O, s), 1558 (N=O, m), 1261 (C-N, N-H, s).

LRMS (ES^{+}): 420 [(M + Na)$^{+}$, 87%], 817 [(2M + Na)$^{+}$, 100%].

HRMS (ES^{+}): $C_{19}H_{31}N_3NaO_6$ (M + Na)$^{+}$: calculated 420.2105, found 420.2106.

H-*L*-Pro-*L*-Ala-*L*-Pro-OMe 99

Compound **99** was prepared following general procedure section 5.2.2 (1.0 mmol). The title compound was obtained as colorless oil and was used in the next step without further purification (0.36 g, 87%).



^{1}H NMR (400 MHz, d^{6}-DMSO): δ 9.96 (1NH-13, br); 8.80 (1NH-8, d, *J* = 8.0 Hz); 8.51 (1NH-13', br); 4.53 (1H-6, apparent p, *J* = 8.0 Hz); 4.30 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.26 (1H-9, m); 3.59 and 3.64 (3H-1, 2 x s, there are two singlets from rotamers); 3.56 (2H-12, m); 3.22 (2H-5, m); 2.25 (2H-10, m); 2.23 (2H-3, m); 1.80-1.92 (2H-11, 2H-4, m); 1.24 and 1.20 (3H-7, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.44 (C), 171.43 (C), 169.09 (C), 59.95 (CH), 59.78 (CH), 52.87 (OCH$_3$-1), 48.60 (CH), 47.65 (CH$_2$), 46.93 (CH$_2$), 30.80 (CH$_2$), 29.68 (CH$_2$), 25.87 (CH$_2$), 24.74 (CH$_2$), 17.49 (CH$_3$-7). There are extra peaks from rotamers at 173.51 (C), 171.73 (C), 168.61 (C), 60.08 (CH), 53.43 (OCH$_3$-1), 46.53 (CH$_2$), 29.21 (CH$_2$), 24.34

(CH_2), 19.16 (CH_3-7) and HOBt at 126.47 (CH), 123.35 (CH), 119.47 (CH), 115.57 (CH), 111.69 (CH) included.

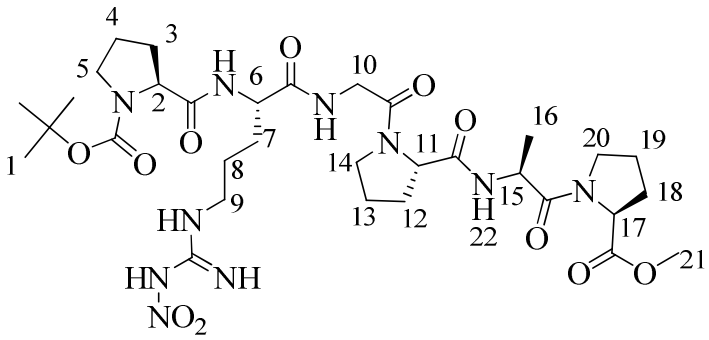
IR (cm^{-1}): 2958 (C-H, br), 1743 (C=O, m), 1651 (C=O, s), 1366 (N-H, C-N, m), 1230 (C-N, N-H, s).

LRMS (ES^+): 298 [$(M + H)^+$, 100%], 595 [$(2M+H)^+$, 12%].

HRMS (ES^+): $C_{14}H_{24}N_3O_4$ $(M + H)^+$: calculated 298.1761, found 298.1766.

N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe 100

Compound **100** was prepared following general procedure section 5.2.1 (1.0 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.38 g, 51%).



m.p.: 67 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.75 (1NH, br); 8.42 (1NH-22, d, *J* = 8.0 Hz); 8.11 (1NH, d, *J* = 8.0 Hz); 4.46 (1H-15, apparent p, *J* = 8.0 Hz); 3.79-4.57 (1H-2, 1H-6, 2H-10, 1H-11, 1H-17, m); 3.59 and 3.60 (3H-21, s, there are two singlets from rotamers), 3.10-3.99 (2H-5, 2H-9, 2H-14, 2H-20, m); 1.54-2.20 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-18, 2H-19, m); 1.30 and 1.37 (9H-1, s, there are two singlets from rotamers); 1.20 and 1.26 (3H-16, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers).

^{13}C NMR (100 MHz, d^6-DMSO): δ 173.40 (C), 173.28 (C), 172.47 (C), 172.01 (C), 171.51 (C), 167.61 (C), 160.27 (C), 154.32 (C), 79.39 ($C(CH_3)_3$), 60.22 (CH), 60.18 (CH), 59.42 (CH), 54.44 (CH), 52.70 (OCH_3-21), 47.46 (CH_2), 47.28 (CH_2), 47.07 (CH), 46.79 (CH_2), 42.72 (CH_2), 42.18 (CH_2), 41.20 (CH_2), 32.00 (CH_2), 30.09 (CH_2), 29.46 (CH_2), 28.94 ($C(CH_3)_3$), 25.63 (CH_2), 25.14 (CH_2), 24.01 (CH_2), 23.06 (CH_2), 17.58 (CH_3-16). There are extra peaks from rotamers at 173.07 (C), 171.39 (C), 167.81 (C), 167.51 (C), 167.20 (C),

154.73 (C), 79.66 ($C(CH_3)_3$), 52.95 (OCH_3-21), 47.74 (CH_2), 32.68 (CH_2), 30.71 (CH_2), 29.05 ($C(CH_3)_3$) and 18.94 (CH_3-16) included.

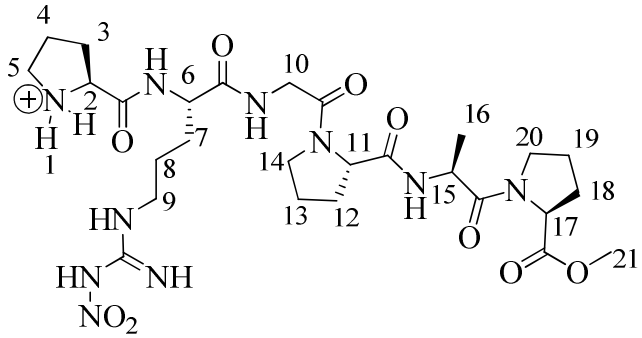
IR (cm^{-1}): 3292 (N-H, br), 2970 (C-H, br), 1740 (C=O, m), 1622 (C=O, s), 1538 (N=O, m), 1366 (N-H, C-N, s), 1252 (C-N, N-H, s).

LRMS (ES^+): 776 [$(M + Na)^+$, 100%].

HRMS (ES^+): $C_{32}H_{53}N_{10}O_{11}$ $(M + H)^+$: calculated 753.3895, found 753.3881.

H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe 101

Compound **101** was prepared following general procedure section 5.2.3 (0.14 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.09 g, 92%).



m.p.: 90 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 9.79 (1NH, br); 8.76 (1NH, t, *J* = 6.0 Hz); 8.50-8.59 (2NH, br); 7.91-8.13 (3NH, br); 4.46 (1H-15, apparent p, *J* = 8.0 Hz); 3.61-4.56 (1H-2, 1H-6, 2H-10, 1H-11, 1H-17, m); 3.60 and 3.63 (3H-21, 2 x s, there are two singlets from rotamers); 3.16-3.53 (2H-5, 2H-9, 2H-14, 2H-20, m); 1.55-2.30 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-18, 2H-19, m); 1.18 and 1.10 (3H-16, d, *J* = 8.0 Hz, there are two doublets from rotamers). There is one N-H proton from peptide chain missing from 1H-spectrum.

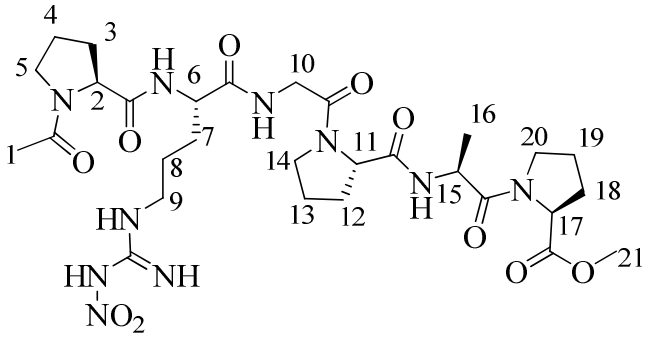
LRMS (ES^+): 338 [$(M+H+Na)^{2+}$, 1%], 653 [$(M+H)^+$, 63%], 675 [$(M+Na)^+$, 77%].

HRMS (ES^+): $C_{27}H_{45}N_{10}O_9$ $(M + H)^+$: calculated 653.3365, found 653.3356.

***N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe 102**

Compound **102** was prepared following general procedure section 5.2.5 (0.13 mmol). All solvents were removed under vacuum and crude residue was purified by column

chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2). The product was precipitated by CH_2Cl_2, MeOH and Et_2O to obtain the title compound as a white solid (0.05 g, 62%).



m.p.: 67 ˚C

^{1}H NMR (300 MHz, $CDCl_3$): δ 11.86 (1NH, br); 9.37 (1NH, t, *J* = 6.0 Hz); 7.57 (1NH, t, *J* = 8.0 Hz) 7.34 (1NH, br); 7.17 (1NH, d, *J* = 8.0 Hz); 3.93-4.61 ((1H-2, 1H-6, 2H-10, 1H-11, 1H-15, 1H-17, m); 3.65 (3H-21, s); 3.05-3.70 (2H-5, 2H-9, 2H-14, 2H-20, m); 2.23 (3H-1, s); 1.62-2.26 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-18, 2H-19, m); 1.30 (3H-16, d, *J* = 8.0 Hz). There is one N-H proton missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, $CDCl_3$): δ 172.31 (C), 172.24 (C), 172.15 (C), 171.99 (C), 171.28 (C), 170.53 (C), 168.40 (C), 154.87 (C), 60.41 (CH), 60.32 (CH), 58.85 (CH), 52.25 (CH), 52.06 (OCH_3-21), 48.42 (CH), 46.91 (CH_2), 46.87 (CH_2), 46.48 (CH_2), 46.23 (CH_2), 42.00 (CH_2), 41.34 (CH_2), 41.20 (CH_2), 28.89 (CH_2), 28.73 (CH_2), 28.44 (CH_2), 25.04 (CH_2), 24.89 (CH_2), 24.60 (CH_2), 22.44 (CH_3-1), 17.29 (CH_3-16). There are extra peaks from rotamers at 171.21 (C), 154.78 (C), 60.24 (CH), 56.84 (CH), 52.67 (CH), 45.79 (CH_2) and 24.89 (CH_2) included

IR (cm^{-1}): 3272 (N-H, br), 2953 (C-H, br), 1738 (C=O, m), 1622 (C=O, s), 1539 (N=O, m), 1373 (N-H, C-N, m), 1266 (C-N, N-H, s).

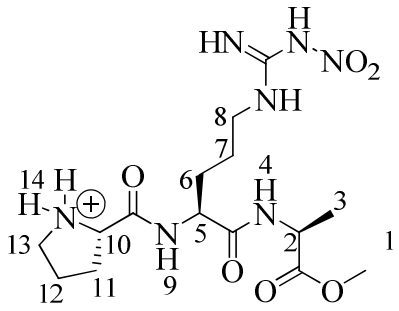
LRMS (ES$^+$): 370 [$(M+2Na)^{2+}$, 4%], 717 [$(M+Na)^+$, 100%], 1411 [$(2M+Na)^+$, 4%].

HRMS (ES$^+$): $C_{29}H_{46}N_{10}NaO_{10}$ $(M + Na)^+$: calculated 717.321, found 717.3297.

f) Synthesis of Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe 106.

H-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe 103

Compound **103** was prepared following general procedure section 5.2.2 (1.0 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.46 g, 89%).



m.p.: 92 ˚C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 9.49 (1NH-14, br); 8.68 (1NH-9, d, *J* = 8.0 Hz); 8.48-8.50 (1NH-4, d, *J* = 8.0 Hz and 1NH-14, br); 7.98 (1NH, br); 4.34 (1H-5, m); 4.26 (1H-2, apparent p, *J* = 8.0 Hz); 4.23 (1H-10, m); 3.61 (3H-1, s); 3.18-3.20 (2H-13, 2H-8, m); 2.28 (2H-11, m); 1.82 (2H-12, m); 1.80 (2H-6, m); 1.56 (2H-7, m); 1.28 (3H-3, d, *J* = 8.0 Hz). There are 2 x N-H protons from guanidine side chain missing from ^{1}H spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.77 (C), 171.72 (C), 169.01 (C), 159.91 (C), 59.74 (CH), 53.30 (CH), 52.80 (OCH_3-1), 48.54 (CH), 46.69 (CH_2), 41.15 (CH_2), 30.51 (CH_2), 30.26 (CH_2), 24.39 (CH_2), 17.72 (CH_3-3). There is one CH_2 missing because the overlapping of CH_2 peaks in ^{13}C spectrum and there are extra peaks from rotamers at 160.26 (C), 159.59 (C), 159.27 (C) and 158.95 (C) included.

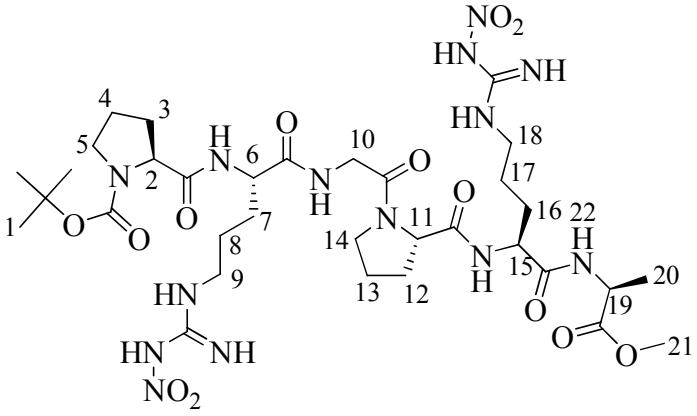
IR (cm^{-1}): 2970 (C-H, br), 1738 (C=O, s), 1652 (C=O, m), 1539 (N=O, m), 1366 (N-H, C-N, s), 1228 (C-N, N-H, s).

LRMS (ES^{+}): 402 [$(M + H)^{+}$, 100%], 803 [$(2M+H)^{+}$, 5%]

HRMS (ES^{+}): $C_{15}H_{28}N_7O_6$ $(M + H)^{+}$: calculated 402.2096, found 402.2095.

N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe 104

Compound **104** was prepared following general procedure section 5.2.1 (2.0 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.72 g, 48 %).



m.p.: 81 ˚C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.52 (1NH, br); 8.40 (1NH, d, *J* = 8.0 Hz); 8.28 (1NH, d, *J* = 8.0 Hz); 8.14 (1NH, t, *J* = 6.0 Hz); 7.97 (1NH-22, d, *J* = 8.0 Hz); 7.92-7.95 (2NH, br); 4.26 (1H-19, apparent p, *J* = 8.0 Hz); 3.85-4.49 (1H-2, 1H-6, 2H-10, 1H-11, 1H-15, m); 3.60 (3H-21, s); 3.15-3.56 (2H-5, 2H-9, 2H-14, 2H-18, m); 1.51-2.09 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-16, 2H-17, m); 1.30 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers); 1.28 (3H-20, d, *J* = 8.0 Hz). There are 3 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, DMSO): δ 172.84 (C), 171.48 (C), 171.37 (C), 167.02 (C), 166.26 (C), 161.21 (C), 159.23 (C), 153.29 (C), 152.04 (C), 78.40 (*C*(CH$_3$)$_3$), 59.22 (CH), 54.89 (CH), 51.80 (OCH$_3$-21), 47.60 (CH), 47.51 (CH), 47.20 (CH$_2$), 46.89 (CH$_2$), 46.78 (CH$_2$), 46.67 (CH$_2$), 46.47 (CH$_2$), 45.96 (CH$_2$), 44.48 (CH$_2$), 30.97 (CH$_2$), 29.34 (CH$_2$), 29.22 (CH$_2$), 29.04 (CH$_2$), 27.99 (C(*C*H$_3$)$_3$), 24.20 (CH$_2$), 23.01 (CH$_2$), 16.76 (CH$_3$-20). There are extra peaks from rotamers at 51.61 (OCH$_3$), 31.81 (CH$_2$), 31.42 (CH$_2$), 29.02 (CH$_2$), 28.09 (C(*C*H$_3$)$_3$), 25.70 (CH$_2$), 24.77 (CH$_2$), 24.68 (CH$_2$), 24.57 (CH$_2$) and 23.92 (CH$_2$) included.

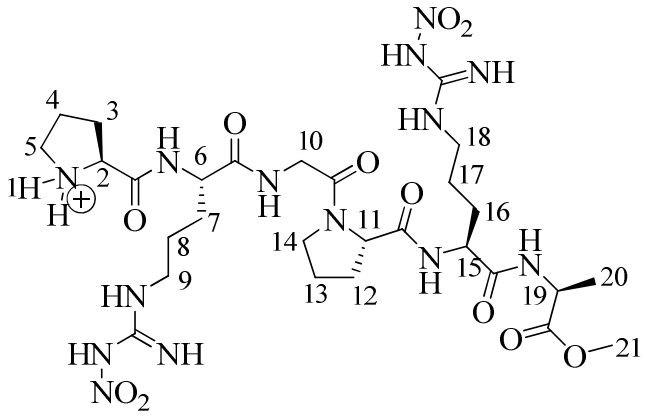
IR (cm^{-1}): 3294 (N-H, br), 2953 (C-H, br), 1743 (C=O, m), 1651 (C=O, s), 1537 (N=O, m), 1393 (N-H, C-N, m), 1257 (C-N, N-H, s).

LRMS (ES$^+$): 451 [(M+2Na)$^{2+}$, 17%], 879 [(M+Na)$^+$, 100%].

HRMS (ES$^+$): $C_{33}H_{56}N_{13}NaO_{14}$ (M + Na$^+$): calculated 879.4043, found 879.4053.

H-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Ala-OMe 105

Compound **105** was prepared following general procedure section 5.2.3 (0.12 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (0.09 g, 95%).



m.p.: 97 ˚C

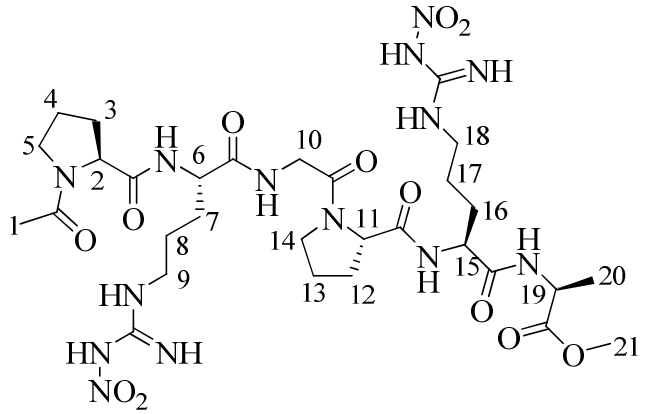
^{1}H NMR (400 MHz, d^6-DMSO): δ 9.21 (1NH, br); 8.68 (1NH, d, *J* = 8.0 Hz); 8.52-8.54 (2NH, br); 8.40 (1NH, d, *J* = 8.0 Hz); 8.28 (1NH, d, *J* = 8.0 Hz); 8.17 (1NH, d, *J* = 4.0 Hz); 8.14 (1NH, br); 8.01 (1NH, d, *J* = 8.0 Hz); 4.26 (1H-19, apparent p, *J* = 8.0 Hz); 4.20-4.43 (1H-2, 1H-6, 2H-10, 1H-11, 1H-15, m); 3.60 (3H-12, s); 3.16-3.53 (2H-5, 2H-9, 2H-14, 2H-18, m); 1.51-2.32 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-16, 2H-17, m); 1.27 (3H-20, d, *J* = 8.0 Hz). There is one N-H proton missing from ^{1}H spectrum.

LRMS (ES$^+$): 757 [(M+H)$^+$, 100%], 779 [(M+Na)$^+$, 13%].

HRMS (ES$^+$): $C_{28}H_{49}N_{14}O_{11}$ (M + H)$^+$: calculated 757.3700, found 757.3688.

N-Ac-*L*-Pro-*L*-Arg(NO$_2$)-Gly-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Ala-OMe 106

Compound **106** was prepared following general procedure section 5.2.5 (0.12 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2). The product was precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (0.06 g, 95%).



m.p.: 67 ˚C

^{1}H NMR (400 MHz, $CDCl_3$): δ 11.94 (1NH, br); 9.36 (1NH, br); 7.56 (1NH, d, *J* = 8.0 Hz); 7.38 (1NH, br); 7.15 (1NH, d, *J* = 8.0 Hz); 4.52 (1H-19, apparent p, *J* = 8.0 Hz); 4.02-4.64 (1H-2, 1H-6, 2H-10, 1H-11, 1H-15, m); 3.64 (3H-21, s); 3.35-3.82 (2H-5, 2H-9, 2H-14, 2H-18, m); 2.24 (3H-1, s); 1.65-2.27 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-16, 2H-17, m); 1.29 (3H-20, d, *J* = 8.0 Hz). There are 5 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.06 (C), 172.27 (C), 172.16 (C), 171.59 (C), 171.45 (C), 170.80 (C), 170.21 (C), 168.51 (C), 154.74 (C), 60.94 (CH), 60.84 (CH), 59.38 (CH), 52.71 (OCH_3-21), 50.70 (CH), 48.81 (CH), 48.85 (CH_2), 47.49 (CH_2), 47.14 (CH_2), 42.56 (CH_2), 41.86 (CH_2), 29.34 (CH_2), 29.27 (CH_2), 29.04 (CH_2), 28.91 (CH_2), 25.38 (CH_2), 25.18 (CH_2), 24.67 (CH_2), 22.93 (CH_3-1), 21.97 (CH_2), 18.06 (CH_3-20).

IR (cm^{-1}): 3273 (br), 2953 (br), 1735 (m), 1623 (s), 1558 (N=O, m), 1347 (N-H, C-N, m), 1275 (C-N, N-H, s).

LRMS (ES^+): 422 [$(M+2Na)^{2+}$, 2%], 443 [$((M+Ac)+2Na)^{2+}$, 16%], 821 [$(M+Na)^{+}$, 1%], 863 [$((M+Ac)+Na)^{+}$, 22%].

HRMS (ES^+): $C_{30}H_{50}N_{14}NaO_{12}$ $(M + Na)^{+}$: calculated 821.3625, found 821.3609.

5.4) EXPERIMENTAL PART FOR CHAPTER 3

5.4.1) General techniques

Peptides were synthesized manually using standard Fmoc (fluorenylmethyloxy carbonyl) chemistry and performed in three ways filtration tubes. Rink amide resin (NovaBiochem) was chosen for solid support in this work and was first treated with 20% piperidine in DMF to remove the Fmoc protecting group and calculated % resin loading by quantitative ninhydrin test. All Fmoc-protected amino acids in this work were *L*-configuration and purchased from NovaBiochem except Fmoc-*L*-Arg(Pbf)-OH was used from Aldrich without further purification. General couplings were mediated with either HOBt/DIC or HBTU/HOBt in the presence of DIPEA in DMF. 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) groups was employed for *L*-arginine residue side chain protection. Fmoc deprotection was accomplished with two treatments of 20% piperidine in DMF. Both coupling and Fmoc deprotection were monitored with either the Kaiser Ninhydrin test (primary *L*-amino acid residue) or 2,3,5,6-tetrachlorobenzoquinone (chloranil, secondary *L*-amino acid residue). The *N*-terminus of peptide was then acetylated with 50% Ac_2O in

pyridine. The mixture of TFA/TIS/H_2O (96:2:2) was used for cleavage of peptide from resin and removal of side-chain protecting group. The crude residue was purified by flash column chromatography and the desired product was precipitated by MeOH/ Et_2O. HOBt, HBTU, DIC and DIPEA were used from Molekular, NovaBiochem, Fluka and Aldrich respectively. TFA and TIS were purchased from Aldrich and used without purification. Piperidine, DMF, Ac_2O (Aldrich) and pyridine were distilled under reduced pressure and calcium hydride before use.

5.4.2) General Procedure for solid phase peptide synthesis

a) Deprotection of Fmoc on resin

Rink amide resins were pre-swollen in CH_2Cl_2 for 15 minutes. 20% Piperidine in DMF (about 20 mL per g of resin) was added to the resin and suspended in the vessel for 20 minutes. The resin was drained and washed with CH_2Cl_2 (20 mL per g of resin x 3), MeOH (20 mL per g of resin x 3), Et_2O (20 mL per g of resin x 3) and 50% DIPEA/DMF (20 mL per g of resin)**.** Each washing was allowed to stand for one minute before draining.

b) Quantitative ninhydrin test

Reagent A:

Solution 1: To a stirred solution of 40 mL of reagent grade phenol and 10 mL of hot absolute ethanol was added 4 g of amberlite mixed-bed resin MB-3 and stirred for 1 h. The resin was then removed by suction filtration.
Solution 2: To a stirred solution of KCN 1.3 mg in 2.0 mL of H_2O was made up volume with distilled pyridine to 100 mL and amberlite mixed-bed resin MB-3 4 g was added. This solution was stirred at room temperature for 1 h and the resin was removed by suction filtration. The solution 1 and 2 were mixed together to obtain reagent A.

Reagent B:

Ninhydrin 25 g was dissolved in 50 mL of absolute ethanol and stored in the dark.

Method: To a known quantity of resin (3-5 mg) that was contained in two small test tubes was added 7 drops of reagent A and 3 drops of reagent B. A control was prepared using the

stains without the resin present. Both test tubes were heated at 100 °C for 5 mins. A solution in each test tube was transferred to 10 mL of volumetric flask and made up volume by 60% solution of ethanol in water. The UV absorbance of both solutions was measured at 570 nm. The control sample was used as blank to run the background. The loading of the resin was calculated using the following formula:

Substitution (mmol/g)	=	$(A_{570} \times V \times 10^3)/ (\varepsilon_{570} \times W)$
ε_{570} (1.5×10^4 $M^{-1}cm^{-1}$)	=	the extinction coefficient of the piperidyl-fulvene adduct at λ 570 nm
V	=	the final volume (mL)
W	=	the weight of the resin sample (mg)
A_{570}	=	the measured absorbance

c) Chloranil test

A few mg of resin beads was placed in a test tube and added 1 drop of 2% chloranil (2,3,5,6-tetrachloro-*p*-benzoquinone) in DMF. A control was prepared using the stains without the resin present. Both of the test tubes were heated for 5 min at 100 ºC. The staining intensity was monitored visually on a white background. The beads contained free amino functions appeared as deep blue color (positive result) while completely coupled beads remained colorless (negative result).

d) General coupling of Fmoc-*L*-amino acids

The resin with a free amine moiety (1.0 mmol of resin loading) was pre-swollen in CH_2Cl_2 for 20 min. To a stirred solution of Fmoc-*L*-amino acid (3.0 equiv, 3.0 mmol) in DMF (2 mL for 3.0 mmol preparation) was slowly added HOBt (3.0 equiv, 3.0 mmol) and DIC (3.0 equiv, 3.0 mmol), respectively. DIPEA (5.0 equiv, 5.0 mmol) was added to the resin, and then the activated amino acid solution was added to the resin. The vessel was sealed and shaken for 7 h. All solvents were removed by filtration and the resin was washed with DMF (20 mL per g of resin x 3), CH_2Cl_2 (20 mL per g of resin x 3), MeOH (20 mL per g of resin x 3) and Et_2O (20 mL per g of resin x 3). Each washing was allowed to stand for one minute before draining.

e) Coupling of Fmoc-*L*-Arg(Pbf)-OH

The resin with a free amine moiety (1.0 mmol of resin loading) was pre-swollen in CH_2Cl_2 for 20 min. To a stirred solution of Fmoc-*L*-Arg(Pbf)-OH (3.0 equiv, 3.0 mmol) in DMF (2 mL for 3.0 mmol preparation) was slowly added HOBt (3.0 equiv, 3.0 mmol) and HBTU (3.0 equiv, 3.0 mmol), respectively. DIPEA (5.0 equiv, 5.0 mmol) was added to the resin, and then the activated amino acid solution was added to the resin. The vessel was sealed and shaken for 7 h. All solvents were removed by filtration and the resin was washed with DMF (20 mL per g of resin x 3), CH_2Cl_2 (20 mL per g of resin x 3), MeOH (20 mL per g of resin x 3) and Et_2O (20 mL per g of resin x 3). Each washing was allowed to stand for one minute before draining.

f) Fmoc-deprotection

The resin with Fmoc protection group (1.0 mmol of resin loading) was added 20% piperidine in DMF (20 mL per g of resin) and shaken for 30 minutes. All solvents were removed by filtration and the resin was washed with DMF (20 mL per g of resin x 3), CH_2Cl_2 (20 mL per g of resin x 3), MeOH (20 mL per g of resin x 3) and Et_2O (20 mL per g of resin x 3). Each washing was allowed to stand for one minute before draining.

g) *C*-terminus Acetylation

Free amine resin (1.0 mmol of resin loading) was added 50% Ac_2O in pyridine (3 equiv, 3.0 mmol) and shaken for 45 min. All solvents were removed by filtration and the resin was washed with DMF (20 mL per g of resin x 3), CH_2Cl_2 (20 mL per g of resin x 3), MeOH (20 mL per g of resin x 3) and Et_2O (20 mL per g of resin x 3). Each washing was allowed to stand for one minute before draining.

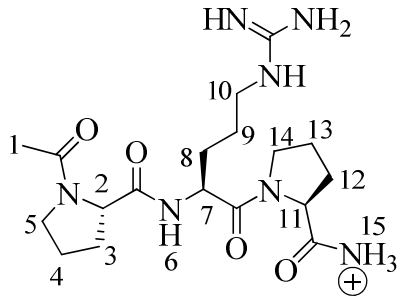
h) Peptide cleavage from rink amide resin

Peptide-resin (1.0 mmol of resin loading) was suspended into mixture of TFA/TIS/H_2O (96:2:2, 20 mL per 1 g of resin). The vessel was sealed and shaked occasionally over 2 h. All solvents were collected by suction filtration. The reaction was repeated one more time with fresh portions of reagents. The combine filtrates were collected by suction filtration and resin was washed with CH_2Cl_2 ((20 mL per g of resin x 3), MeOH ((20 mL per g of

resin x 3) and Et_2O (20 mL per g of resin x 3). Each washing was allowed to stand for one minute before draining. All solvents were removed by toluene azeotrope *in vacuo*. The desired product was purification by flash column chromatography and precipitated by MeOH and diethyl ether.

5.4.3) Synthesis of model compound: *N*-Ac-*L*-Pro-*L*-Arg-*L*-Pro-NH_2 119.

The synthesis of compound **119** was manually synthesized following general procedure section 5.5.2 (300 mg of resin loading, 0.22 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH. Tripeptide was cleaved from resin support and desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (75 mg, 84%).



m.p. 95 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.49 (1NH, d, *J* = 4.0 Hz); 8.09 (1NH-6, d, *J* = 8.0 Hz) 7.80 (1NH, br); 7.32 (3NH-15, br); 6.86 (1NH, br); 4.46 (1H-7, m); 4.19-4.32 (1H-2, 1H-11, m); 3.04-3.62 (2H-5, 2H-10, 2H-14, m); 1.96 (3H-1, s); 1.52-2.02 (2H-3, 2H-4, 2H-8, 2H-9, 2H-12, 2H-13, m).

^{13}C NMR (100 MHz, d^4-MeOD): δ 173.91 (C), 171.94 (C), 169.80 (C), 168.73 (C), 156.98 (C), 59.97 (CH), 59.35 (CH), 50.39 (CH), 48.09 (CH_2), 47.06 (CH_2), 46.77 (CH_2), 40.89 (CH_2), 29.97 (CH_2), 29.71 (CH_2), 29.94 (CH_2), 24.77 (CH_2), 23.01 (CH_2), 22.73 (CH_3-Ac). There are extra peaks from rotamer at 173.81 (C), 172.31 (C), 169.97 (C), 157.08 (C), 60.35 (CH), 50.80 (CH), 24.99 (CH) and 22.50 (CH_3-Ac) included.

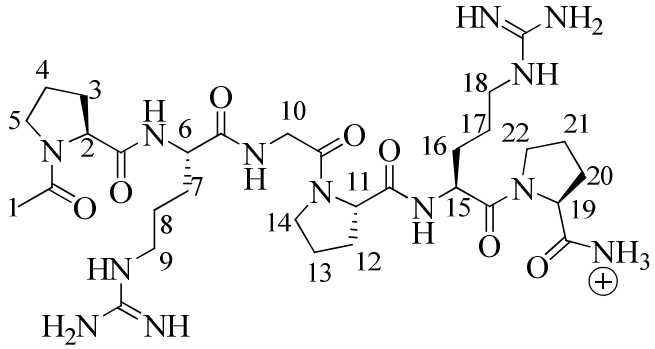
IR (cm^{-1}): 3183 (N-H, br), 1633 (C=O, s), 1447 (N-H, C-N, s), 1200 (C-N, N-H, s).

LRMS (ES$^+$): 410 [$(M+H)^+$, 100%].

HRMS (ES$^+$): $C_{18}H_{32}N_7O_4$ $(M + H)^+$: calculated 410.2510, found 410.2518.

5.4.4) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 1.

The synthesis of compound **1** was manually synthesized following general procedure section 6.5.2 (400 mg of resin loading, 0.28 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Pro-OH, Fmoc-Gly-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH. Finally, hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg-Gly-*L*-Pro-*L*-Arg-*L*-Pro-NH_2 **53,** was cleaved from resin support and purified by flash column chromatography (25% CH_3CN/H_2O). The desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (65 mg, 32%).



m.p. 101 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.46 (1NH, br); 8.19 (1NH, d, *J* = 8.0 Hz); 7.58-8.12 (3NH, br) 6.87-7.30 (7NH, br); 3.71-4.53 (1H-2, 1H-6, 2H-10, 1H-11, 1H-15, 1H-19, m); 3.04-3.57 (2H-5, 2H-9, 2H-14, 2H-18, 2H-22, m); 1.98 (3H-1, s); 1.51-2.03 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-16, 2H-17, 2H-20, 2H-21, m). There are 2 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^4-MeOD): δ 168.50 (C), 166.49 (C), 165.90 (C), 165.79 (C), 164.24 (C), 164.20 (C), 163.59 (C), 161.05 (C), 150.01 (C), 53.05 (CH), 52.95 (CH), 52.89 (CH), 49.76 (CH_2), 45.54 (CH), 43.58 (CH), 41.09 (CH_2), 40.15 (CH_2), 39.37 (CH_2), 34.22 (CH_2), 33.54 (CH_2), 33.38 (CH_2), 24.75 (CH_2), 22.59 (CH_2), 22.41 (CH_2), 22.03 (CH_2), 21.35 CH_2), 20.74 (CH_2), 17.45 (CH_2), 17.33 (CH_2), 17.27 (CH_2), 13.93 (CH_3-1). There are extra peaks from rotamers at 52.13 (CH), 46.27 (CH_2), 41.17 (CH_2), 40.95 (CH_2), 40.74 (CH_2), 40.53 (CH_2), 40.44 (CH_2), 40.23 (CH_2) and 24.81 (CH_2) included.

IR (cm^{-1}): 3292 (N-H, br), 1627 (C=O, s), 1447 (N-H, C-N, m), 1189 (C-N, N-H, m).

LRMS (ES$^+$): 360 [$(M+2H)^{2+}$, 100%].

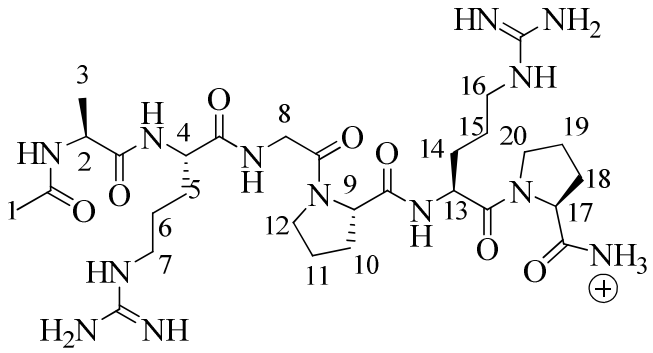
HRMS (ES$^+$): $C_{31}H_{54}N_{13}O_7$ $(M + H)^+$: calculated 720.4264, found 720.4262.

LCMS (ES^+): 720.4 [$(M+H)^+$, 100%].

5.4.5) Synthesis of *L*-alanine scanning peptide 79-84 by solid phase peptide synthesis.

a) Synthesis of *N*-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 120.

The synthesis of compound **120** was manually synthesized following general procedure section 5.5.2 (300 mg of resin loading, 0.21 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Pro-OH, Fmoc-Gly-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Ala-OH. Finally, hexapeptide, *N*-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **79,** was cleaved from resin support and purified by flash column chromatography (25% CH_3CN/H_2O). The desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (69 mg, 47%).



m.p. 110 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.20 (1NH, t, *J* = 8.0 Hz); 8.16 (1NH, d, *J* = 8.0 Hz); 7.15-7.31 (9NH, br); 6.87 (1NH, br); 3.90-4.47 (1H-2, 1H-4, 2H-8, 1H-9, 1H-13, 1H-17, m); 3.02-3.56 (2H-7, 2H-12, 2H-16, 2H-20, m); 1.86 (3H-1, s); 1.49-2.07 (2H-5, 2H-6, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.10 and 1.06 (3H-3, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers). There are 3 x N-H protons missing from ^{1}H-spectrum.

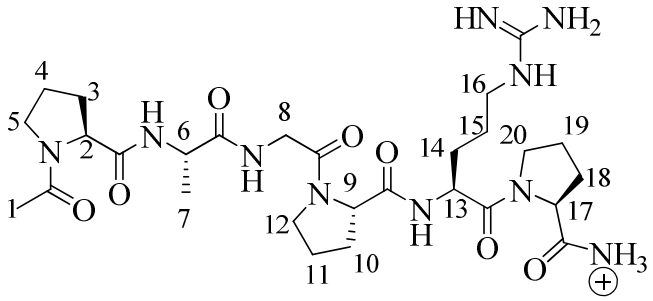
^{13}C NMR (100 MHz, d^4-MeOD): δ 179.47 (C), 174.67 (C), 172.51 (C), 170.92 (C), 168.86 (C), 164.54 (C), 163.04 (C), 157.72 (C), 155.58 (C), 59.74 (CH), 54.93 (CH), 54.86 (CH_2), 52.09 (CH), 49.43 (CH), 48.58 (CH), 46.28 (CH_2), 40.70 (CH_2), 40.34 (CH_2), 39.91 (CH_2), 39.71 (CH_2), 38.83 (CH_2), 32.35 (CH_2), 30.96 (CH_2), 24.86 (CH_2), 24.65 (CH_2), 21.83 (CH_2), 19.38 (CH_2), 22.70 (CH_3-1), 18.14 (CH_3-3).

IR (cm^{-1}): 3293 (N-H, br), 1622 (C=O, s), 1447 (N-H, C-N, m).

LRMS (ES$^+$): 347 [(M+2H)$^{2+}$, 100%], 367 [(M+Na+NH$_4$)$^{2+}$, 40%], 694 [(M+H)$^+$, 2%].
HRMS (ES$^+$): $C_{29}H_{52}N_{13}O_7$ (M + H)$^+$ calculated 694.4107, found 694.4102.
LCMS (ES$^+$): 347.7 [(M+2H)$^{2+}$, 100%].

b) Synthesis of *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH$_2$ 121.

The synthesis of compound **121** was manually synthesized following general procedure section 5.5.2 (300 mg of resin loading, 0.22 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Pro-OH, Fmoc-Gly-OH, Fmoc-*L*-Ala-OH and Fmoc-*L*-Pro-OH. Finally, hexapeptide, *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH$_2$, **121** was cleaved from resin support and desired product was precipitated from MeOH and Et$_2$O to obtain the title compound as a pale yellow solid (43 mg, 31%).

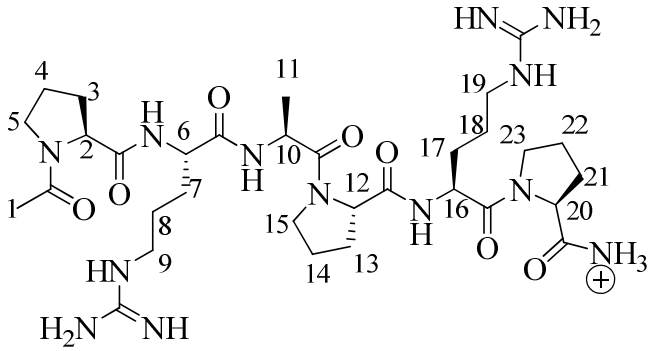


m.p. 87 °C
^{1}H NMR (400 MHz, d^6-DMSO): δ 8.49 (1NH, br); 8.10-8.15 (2NH, br); 7.98 (1NH, br); 7.56 (1NH, br); 7.02-7.31 (3NH, br); 6.85-6.86 (2NH, br); 3.80-4.48 (1H-2, 1H-6, 2H-8, 1H-9, 1H-13, 1H-17, m) 3.29-3.60 (2H-5, 2H-12, 2H-16, 2H-20, m); 1.86 (3H-Ac, s); 1.53-2.09 (2H-3, 2H-4, 2H-10, 2H-11, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.22 (3H-7, d, *J* = 8.0 Hz).
^{13}C NMR (100 MHz, d^4-MeOD): δ 177.44 (C), 175.59 (C), 174.99 (C), 174.81 (C), 173.34 (C), 172.45 (C), 169.88 (C), 158.95 (C), 62.05 (CH), 61.93 (CH), 61.87 (CH), 52.45 (CH), 50.72 (CH), 50.00 (CH_2), 49.08 (CH_2), 48.30 (CH_2), 43.23 (CH_2), 42.48 (CH_2), 33.80 (CH_2), 31.37 (CH_2), 31.03 (CH_2), 29.77 (CH_2), 26.45 (CH_2), 26.43 (CH_2), 26.26 (CH_2), 26.20 (CH_2), 22.89 (CH_3-1), 18.23 (CH_3-7).
IR (cm^{-1}): 3275 (N-H, br), 2970 (C-H, br), 1738 (C=O, m), 1622 (C=O, s), 1445 (N-H, C-N, m), 1202 (C-N, N-H, s).
LRMS (ES$^+$): 635 [(M+H)$^+$, 100%].

HRMS (ES$^+$): $C_{28}H_{47}N_{10}O_7$ $(M + H)^+$ calculated 635.3624, found 635.3637.
LCMS (ES$^+$): 318.2 $[(M+2H)^{2+}$, 100%].

c) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH$_2$ 122.

The synthesis of compound **122** was manually synthesized following general procedure section 5.5.2 (400 mg of resin loading, 0.29 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Pro-OH, Fmoc-*L*-Ala-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH. Finally, hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH$_2$, **122** was cleaved from resin support and purified by flash column chromatography (25% CH_3CN/H_2O). The desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (74 mg, 35%).



m.p. 109 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.23 (1NH, br); 8.04-8.07 (2NH, br); 7.88-7.89 (2NH, br); 7.56-7.67 (2NH, br); 7.22-7.31 (5NH, br); 6.86-6.87 (2NH, br); 4.19-4.46 (1H-2, 1H-6, 1H-10, 1H-12, 1H-16, 1H-20, m); 3.17-3.61 (2H-5, 2H-9, 2H-15, 2H-19, 2H-23, m); 1.86 (3H-1, s); 1.20-2.02 (2H-3, 2H-4, 2H-7, 2H-8, 2H-13, 2H-14, 2H-21, 2H-22, m); 1.18-1.20 (3H-11, d, J = 5.1 Hz).

^{13}C NMR (100 MHz, d^4-MeOD): δ 174.73 (C), 172.94 (C), 172.36 (C), 171.92 (C), 171.70 (C), 170.56 (C), 170.02 (C), 169.69 (C), 157.68 (C), 60.39 (CH), 57.88 (CH), 54.89 (CH), 54.37 (CH), 52.71 (CH), 50.88 (CH), 48.67 (CH_2), 47.76 (CH_2), 47.36 (CH_2), 41.57 (CH_2), 41.35 (CH_2), 41.29 (CH_2), 40.21 (CH_2), 32.60 (CH_2), .30.50 (CH_2), 30.31 (CH_2), 30.00 (CH_2), 25.56 (CH_2), 25.47 (CH_2), 25.36 (CH_2), 24.35 (CH_3-1), 23.54 (CH_2), 17.66 (CH_3-11). There are extra peaks from rotamer at 170.60 (C), 157.90 (C), 157.74 (C), 157.65 (C), 60.30 (CH), 60.25 (CH), 60.17 (CH) and 28.92 (CH_2) included.

IR (cm^{-1}): 3279 (N-H, br), 1622 (C=O, s), 1447 (N-H, C-N, s).

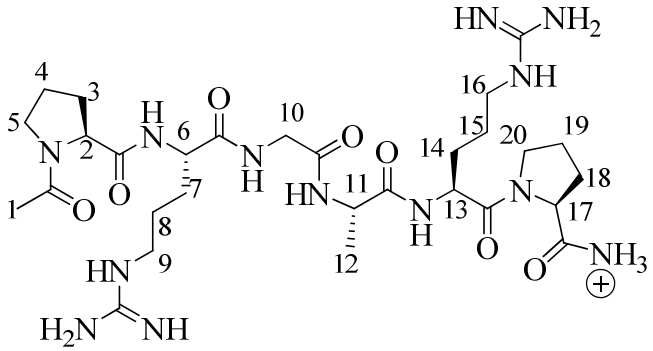
LRMS (ES^+): 367 [$(M+2H)^{2+}$, 100%].

HRMS (ES^+): $C_{32}H_{57}N_{13}O_7$ $(M + 2H)^{2+}$ calculated 367.7246, found 367.7244.

LCMS (ES^+): 367.9 [$(M+2H)^{2+}$, 100%].

d) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2 123.

The synthesis of compound **123** was manually synthesized following general procedure section 5.5.2 (200 mg of resin loading, 0.22 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Ala-OH, Fmoc-Gly-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH. Finally, hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2, **123** was cleaved from resin support and purified by flash column chromatography (25% CH_3CN/H_2O). The desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (59 mg, 39%).



m.p. 91 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.31 (1NH, br); 7.07-8.21 (11NH, br); 3.88-4.55 (1H-2, 1H-6, 2H-10, 1H-11, 1H-13, 1H-17, m); 3.02-3.46 (2H-5, 2H-9, 2H-16, 2H-20, m); 1.85 (3H-1, s); 1.39-2.01 (2H-3, 2H-4, 2H-7, 2H-8, 2H-14, 2H-15, 2H-18, 2H-19, m); 1.10 and 1.07 (3H-12, 2 x d, *J* = 8.0 Hz, there are two doublets from rotamers). There are 2 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^4-MeOD): δ 174.87 (C), 174.28 (C), 173.07 (C), 172.48 (C), 172.18 (C), 172.05 (C), 171.47 (C), 171.33 (C), 157.65 (C), 60.62 (CH), 59.94 (CH), 59.71 (CH), 52.21 (CH), 51.22 (CH), 48.13 (CH_2), 47.00 (CH_2), 46.94 (CH_2), 46.31 (CH_2), 44.20 (CH_2), 41.69 (CH_2), 40.37 (CH_2), 39.84 (CH_2), 37.43 (CH_2), 29.65 (CH_2), 24.90 (CH_2), 24.62 (CH_2), 21.60 (CH_2), 23.39 (CH_3-1), 18.27 (CH_3-12). There are extra peaks from

rotamers at 174.80 (C), 174.19 (C), 57.59 (CH), 51.19 (CH), 23.30 (CH_3-1) and 18.55 (CH_3-12) included.

IR (cm^{-1}): 3275 (N-H, br), 1623 (C=O, s), 1447 (N-H, C-N, m), 1202 (C-N, N-H, m).

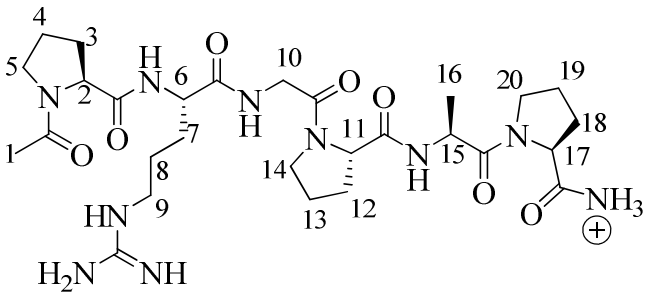
LRMS (ES^+): 347 [$(M+2H)^{2+}$, 100%], 366 [$(M+Na+NH_4)^{2+}$, 2%], 369 [$(M+2Na)^{2+}$, 5%], 694 [$(M+H)^+$, 5%].

HRMS (ES^+): $C_{29}H_{52}N_{13}O_7$ $(M + H)^+$ calculated 694.4107, found 694.4112.

LCMS (ES^+): 694.5 [$(M+H)^+$, 100%].

e) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2 124.

The synthesis of compound **124** was manually synthesized following general procedure section 5.5.2 (300 mg of resin loading, 0.22 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Pro-OH, Fmoc-*L*-Ala-OH, Fmoc-*L*-Pro-OH, Fmoc-Gly-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH. Finally, hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2, **124** was cleaved from resin support and desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (95 mg, 68%).



m.p. 87 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.34 (1NH, br); 8.07-8.10 (2NH, br); 7.87 (1NH, br); 7.51 (1NH, br); 7.42 (1NH, br); 7.18-7.19 (2NH, br); 6.85-6.86 (2NH, br); 3.85-4.56 (1H-2, 1H-6, 2H-10, 1H-11, 1H-15, 1H-17, m); 3.38-3.56 (2H-5, 2H-9, 2H-14, 2H-20, m); 1.77-2.18 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-18, 2H-19, m); 1.87 (3H-1, s); 1.52-1.54 (3H, m); 1.23 and 1.20 (3H-16, 2 x d, J = 8.0 Hz, There are two doublets from rotamers).

^{13}C NMR (100 MHz, d^4-MeOD): δ 167.57 (C), 165.43 (C), 164.49 (C), 163.73 (C), 163.16 (C), 160.00 (C), 154.76 (C), 149.04 (C), 61.98 (CH), 61.90 (CH), 54.31 (CH), 50.04 (CH_2), 49.00 (CH), 48.93 (CH_2), 48.25 (CH_2), 48.10 (CH_2), 43.19 (CH_2), 42.36

(CH_2), 40.59 (CH), 31.54 (CH_2), 31.28 (CH_2), 31.00 (CH_2), 30.43 (CH_2), 26.40 (CH_2), 26.31 (CH_2), 26.12 (CH_2), 22.87 (CH_3-1), 18.65 (CH_3-16). There are extra peaks from rotamers at 54.79 (CH), 49.97 (CH_2), 49.75 (CH), 47.46 (CH_2), 33.65 (CH_2), 26.47 (CH_2) and 17.30 (CH_3-16) included.

IR (cm^{-1}): 3276 (N-H, br), 1618 (C=O, s) 1448 (N-H, C-N, s), 1200 (C-N, N-H, m).

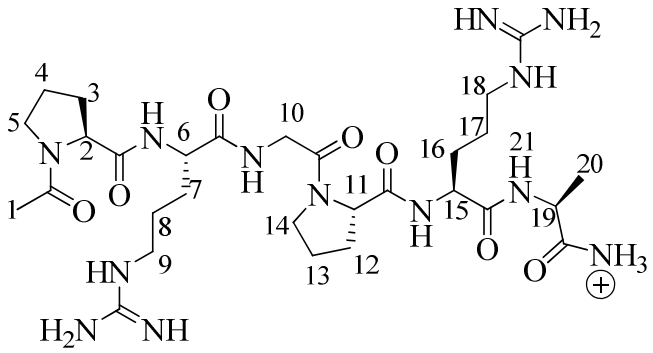
LRMS (ES^+): m/z 635 [$(M+H)^+$, 56%].

HRMS (ES^+): $C_{28}H_{47}N_{10}O_7$ $(M + H)^+$ calculated 635.3624, found 635.3624.

LCMS (ES^+): 635.4 [$(M+H)^+$, 100%].

f) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2 125.

The synthesis of compound **125** was manually synthesized following general procedure section 5.5.2 (200 mg of resin loading, 0.24 mmol). Synthetic procedure was started with step by step coupling and Fmoc-deprotection to the resin. Protected amino acid was subsequently introduced as Fmoc-*L*-Ala-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Pro-OH, Fmoc-Gly-OH, Fmoc-*L*-Arg(Pbf)-OH and Fmoc-*L*-Pro-OH. Finally, hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2, **125** was cleaved from resin support and purified by flash column chromatography (25% CH_3CN/H_2O). The desired product was precipitated from MeOH and Et_2O to obtain the title compound as a pale yellow solid (74 mg, 45%).



m.p. 98°C

^{1}H NMR (400 MHz, d^6-DMSO): δ 6.88-8.12 (11NH, br); 3.56-4.55 (1H-2, 1H-6, 2H-10, 1H-11, 1H-15, 1H-19, m); 3.06-3.36 (2H-5, 2H-9, 2H-14, 2H-18, m); 1.82 (3H-1, s); 1.34-2.16 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-13, 2H-16, 2H-17, m); 1.11 and 1.05 (3H-20, 2 x d, J = 8.0 Hz, there are two doublets from rotamers). There are 4 x N-H protons missing from ^{1}H spectrum.

^{13}C NMR (100 MHz, d^4-MeOD): δ 174.68 (C), 174.57 (C), 172.70 (C), 172.62 (C), 170.88 (C), 170.48 (C), 167.90 (C), 157.83 (C), 157.72 (C), 60.42 (CH), 60.29 (CH), 58.62 (CH), 53.07 (CH), 51.06 (CH), 47.54 (CH_2), 47.45 (CH_2), 46.78 (CH_2), 42.07 (CH_2), 41.50 (CH_2), 30.23 (CH_2), 30.04 (CH_2), 29.57 (CH_2), 29.10 (CH_2), 28.88 (CH_2), 27.54 (CH_2), 26.72 (CH_2), 25.45 (CH_2), 23.37 (CH_3-1), 18.85 (CH_3-20). There are extra peaks from rotamers at 172.41 (C), 170.95 (C), 170.67 (C), 167.79 (C), 60.54 (CH), 60.19 (CH), 50.89 (CH), 46.93 (CH_2), 41.86 (CH_2), 40.72 (CH_2), 40.43 (CH_2), 40.23 (CH_2), 40.02 (CH_2), 23.44 (CH_3-1) included.

IR (cm^{-1}): 3392 (N-H, br), 1624 (C=O, m), 1420 (N-H, C-N, s).

LRMS (ES$^+$): m/z 347 [$(M+2H)^{2+}$, 100%], 694 [$(M+H)^+$, 6%].

HRMS (ES$^+$): $C_{29}H_{52}N_{13}O_7$ $(M + H)^+$ calculated 694.4107, found 694.4113.

LCMS (ES$^+$): 347.9 [$(M+2H)^{2+}$, 100%].

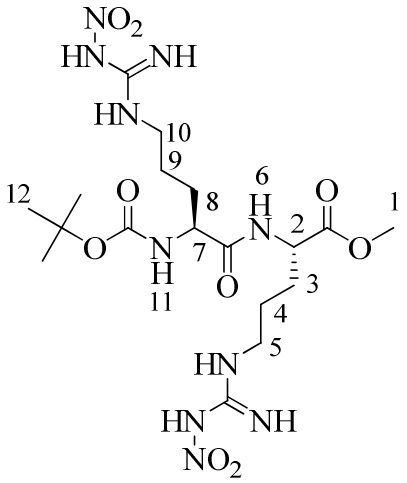
5.5) EXPERIMENTAL PART FOR CHAPTER 4

5.5.1) Synthesis of protection hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe, 150.

Peptides were synthesized by using standard solution phase peptide synthesis and general procedures were achieved in section 5.2.

N-Boc-*L*-Arg(NO_2)-*L*-Arg(NO_2)-OMe 142

Compound **142** was prepared by general procedure 5.2.1 (6.27 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (2.34 g, 70%).



m.p. 67 °C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.50 (1NH, br); 8.20 (1NH-6, d, *J* = 8.0 Hz); 7.85-7.87 (3NH, br); 6.85 (1NH-11, d, *J* = 8.0 Hz); 4.26 (1H-2, apparent dd, *J* = 8.0, 12.0 Hz); 3.93 (1H-7, m); 3.61 (3H-1, s); 3.14 (2H-5, 2H-10, m); 1.51-1.63 (2H-4, 2H-3, 2H-9, 2H-8, m); 1.36 (9H-12, s).

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 181.81 (C), 173.21 (C), 172.20 (C), 160.25 (C), 156.18 (C), 79.04 (*C*$(CH_3)_3$), 54.61 (CH), 52.78 (OCH_3-1), 52.46 (CH), 41.26 (CH_2), 41.03 (CH_2), 33.22 (CH_2), 30.06 (CH_2), 29.10 ($C(CH_3)_3$-12), 29.01 (CH_2), 25.56 (CH_2).

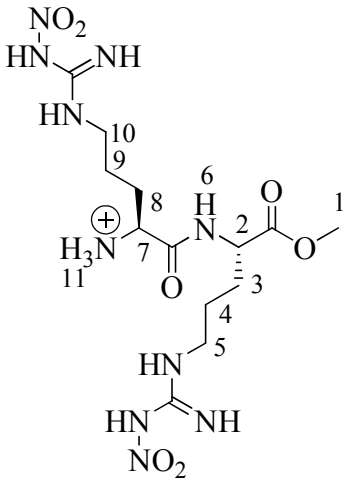
IR (cm^{-1}): 3285 (N-H, br), 2978 (C-H, br), 1741 (O=C, m), 1626 (O=C, s), 1530 (N=O, m), 1391 (N-H, C-N, m), 1251 (C-N, N-H, s).

LRMS (ES^{+}): 557 [$(M + Na)^{+}$, 100%].

HRMS (ES^{+}): $C_{18}H_{34}N_{10}NaO_9$ $(M + Na)^{+}$: calculated 557.2402, found 557.2397.

H-*L*-Arg(NO_2)-*L*-Arg(NO_2)-OMe x HCl 143

Compound **143** was prepared by general procedure 5.2.3 (4.39 mmol). The title compound was obtained as a white solid and was used in the next step without further purification (1.90 g, 92%).



m.p. 92 °C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 9.01 (1NH-6, br); 8.54 (1NH, br); 8.29 (1NH$_3$-11, br); 7.98-7.99 (3NH, br) 4.31 (1H-2, apparent dd, *J* = 8.0, 12.0 Hz); 3.86 (1H-7, m); 3.63 (3H-1, s); 3.16-3.17 (2H-10, 2H-5, m); 1.58-1.78 (2H-3, 2H-4, 2H-8, 2H-9, m). There are 2 x N-H protons from guanidine side chain missing ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 181.81 (C), 172.68 (C), 169.70 (C), 160.24 (C), 55.86 (CH), 52.99 (CH), 52.86 (OCH_3-1), 52.50 (CH), 41.08 (CH_2), 40.98 (CH_2), 40.89 (CH_2), 33.23 (CH_2), 29.33 (CH_2), 28.75 (CH_2).

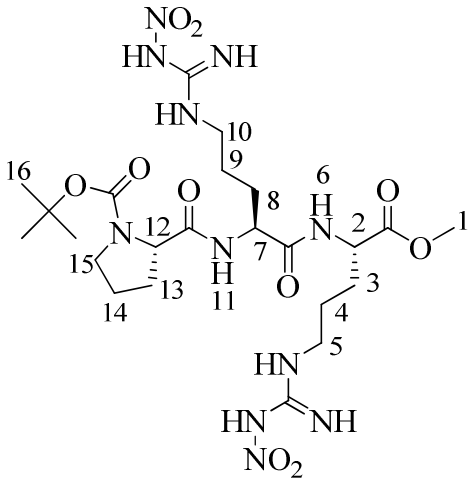
IR (cm^{-1}): 2970 (C-H, br), 1738 (O=C, m), 1678 (O=C, s), 1547 (N=O, m), 1366 (N-H, C-N, m), 1262 (C-N, N-H, s).

LRMS (ES^{+}): 435 [(M + H)$^{+}$, 40%], 869 [(2M + H)$^{+}$, 2%].

HRMS (ES^{+}): $C_{13}H_{27}N_{10}O_7$ (M + H)$^{+}$: calculated 435.2064, found 435.2054.

N-Boc-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Arg(NO$_2$)-OMe 144

Compound **144** was prepared by general procedure 5.2.1 (4.38 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.44 g, 52%).



m.p. 69 °C

^{1}H NMR (400 MHz, d^6-DMSO): δ 9.21 (1NH, br); 8.28-8.51 (2NH, br); 8.38 (1NH-11, d, *J* = 8.0 Hz); 7.88-7.90 (1NH-6, d, *J* = 8.0 Hz and 2NH, br); 4.23-4.30 (1H-12, m and 1H-2, apparent dd, *J* = 8.0, 12.0 Hz); 4.13 (1H-7, m); 3.61 (3H-1, s); 3.09-3.58 (2H-15, 2H-10, 2H-5, m); 1.38-1.77 (2H-3, 2H-4, 2H-8, 2H-9, 2H-13, 2H-14, m); 1.26, 1.28, 1.29 and 1.31 (9H-16, 4 x s, there are four singlets from rotamers). There is one N-H proton from guanidine side chain missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^4-MeOD): δ 175.81 (C), 174.15 (C), 173.69 (C), 161.12 (C), 156.60 (C), 156.07 (C), 81.56 (*C*(CH$_3$)$_3$), 61.46 (CH), 56.01 (CH), 54.51 (CH), 53.05 (OCH$_3$-1), 48.51 (CH$_2$), 48.11 (CH$_2$), 43.98 (CH$_2$), 41.82 (CH$_2$), 32.60 (CH$_2$), 31.52 (CH$_2$), 30.32 (CH$_2$), 29.73 (CH$_2$), 28.89 (C(*C*H$_3$)$_3$), 24.80 (CH$_2$). There are extra peaks from rotamers at 175.52 (C), 66.98 (CH), 53.39 (CH) and 26.22 (CH$_2$) included.

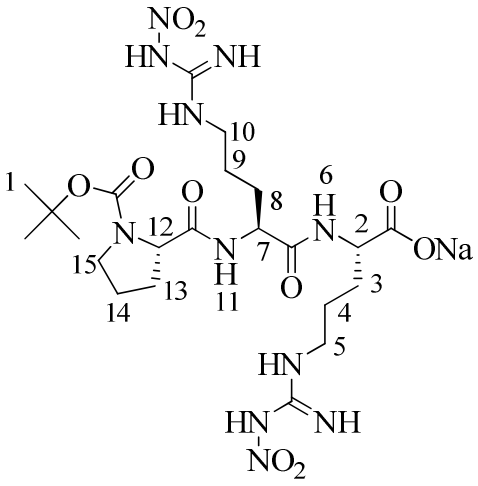
IR (cm^{-1}): 3279 (N-H, br), 2977 (C-H, br), 1742 (O=C, m), 1625 (O=C, s), 1537 (N=O, m), 1392 (N-H, C-N, s), 1253 (C-N, N-H, s).

LRMS (ES$^+$): 654 [(M + Na)$^+$, 10%].

HRMS (ES$^+$): $C_{23}H_{41}N_{11}NaO_{10}$ (M + Na)$^+$: calculated 654.2930, found 654.2931.

N-Boc-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Arg(NO$_2$)-ONa 145

Compound **145** was prepared by general procedure 5.2.4 (2.28 mmol). The title product was obtained as a white solid and was use in the next step without further purification (1.30 g, 87 %).



m.p. 102 °C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.27 (1NH-11, br); 7.52 (1NH-6, br); 4.16-4.17 (1H-12, 1H-2, m); 3.86 (1H-7, m); 3.12-3.40 (2H-15, 2H-10, 2H-5, m) 1.52-2.13 (2H-3, 2H-4, 2H-8, 2H-9, 2H-13, 2H-14, m); 1.30 and 1.37 (9H-1, 2 x s, there are two singlets from rotamers). There are 6 x N-H protons missing from ^{1}H spectrum.

^{13}C NMR (100 MHz, d^{4}-MeOD): δ 178.60 (C), 175.17 (C), 173.27 (C), 161.05 (C), 156.56 (C), 156.03 (C), 81.43 ($C(CH_3)_3$), 61.34 (CH), 55.77 (CH), 54.85 (CH), 48.41 (CH_2), 47.98 (CH_2), 42.18 (CH_2), 41.87 (CH_2), 32.67 (CH_2), 31.13 (CH_2), 30.39 (CH_2), 28.86 ($C(CH_3)_3$), 26.28 (CH_2), 24.72 (CH_2). There are extra peaks from rotamers at 175.62 (C), 161.18 (C), 81.40 ($C(CH_3)_3$), 61.43 (CH), 54.70 (CH), 34.42 (CH_2), 31.51(CH_2), 30.10 (CH_2), 28.95 ($C(CH_3)_3$), 26.66 (CH_2), 25.53 (CH_2) and HOBt at 128.79 (CH), 125.18 (CH), 124.99 (CH), 118.82 (CH) and 112.53 (CH) included.

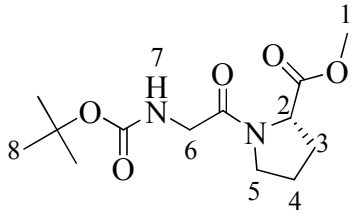
IR (cm^{-1}): 2952 (N-H, br), 2748 (C-H, br), 1739 (O=C, m), 1647 (O=C, s), 1557 (N=O, m), 1365 (N-H, C-N, m), 1259 (C-N, N-H, s).

LRMS (ES^{+}): 662 [$(M + Na)^{+}$, 100%].

HRMS (ES^{+}): $C_{22}H_{38}N_{11}Na_2O_{10}$ $(M + Na)^{+}$: calculated 662.2593, found 662.2586.

N-Boc-Gly-*L*-Pro-OMe 146

Compound **146** was prepared by general procedure 5.2.1 (11.42 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 2% MeOH/CH_2Cl_2) and yielded the title compound as colorless oil (1.86 g, 60%).



^{1}H NMR (400 MHz, d^{6}-DMSO): δ 6.79 (1NH-7, t, *J* = 6.0 Hz); 4.30 and 4.67 (1H-2, 2 x dd, *J* = 5.0, 9.0 Hz, there are two double doublets from rotamers); 3.77 and 3.82 (2H-6, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.61 and 3.70 (3H-1, 2 x s, there are two singlets from rotamers); 3.52 (2H-5, m); 2.14 (1H-3, m); 1.93 (2H-4, m); 1.84 (1H-3', m); 1.38 (9H-8, s).

^{13}C NMR (100 MHz, d^{6}-DMSO): δ 173.34 (C), 168.47(C), 156.65 (C), 78.81 ($C(CH_3)_3$), 59.38 (CH-2), 52.67 (OCH_3-1), 46.38 (CH_2), 43.16 (CH_2), 29.46 (CH_2), 29.10 ($C(CH_3)_3$), 25.36 (CH_2). There are extra peaks from rotamers at 168.68 (C), 58.64 (CH), 53.30 (OCH_3), 47.16 (CH_2), 43.08 (CH_2), 31.77 (CH_2) and 28.80 ($C(CH_3)_3$) included.

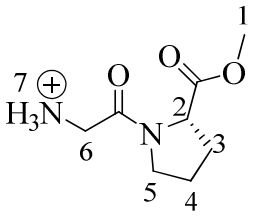
IR (cm^{-1}): 3391 (N-H, br), 2978 (C-H, br), 1740 (O=C, m), 1647 (O=C, s), 1366 (N-H, C-N, s), 1283 (C-N, N-H, m).

LRMS (ES^{+}): 309 [$(M + Na)^{+}$, 100%], 595 [$(2M+Na)^{+}$, 92%].

HRMS (ES^{+}): $C_{13}H_{22}N_2NaO_5$ $(M + Na)^{+}$: calculated 309.1421, found 309.1419.

H-Gly-*L*-Pro-OMe x HCl 147

Compound **147** was prepared by general procedure 5.2.3 (6.86 mmol). The title product was obtained as colorless oil and was use in the next step without further purification (1.21 g, 95%).



^{1}H NMR (400 MHz, d^{6}-DMSO): δ 8.42 ($1NH_3$-7, br); 4.30 and 4.67 (1H-2, 2 x dd, *J* = 5.0, 9.0 Hz, there are two double doublets from rotamers); 3.79 (2H-6, m); 3.62 and 3.69 (3H-1, 2 x s, there are two singlet from rotamers); 3.54 (2H-5, m); 2.16 (2H-3, m); 1.92 (2H-4, m).

^{13}C NMR (100 MHz, d^{6}-DMSO): 172.93 (C), 165.64 (C), 59.49 (CH-2), 52.96 (OCH_3-1), 46.62 (CH_2), 40.61 (CH_2), 29.68 (CH_2), 25.21 (CH_2). There are extra peaks from rotamers

at 173.68 (C), 172.89 (C), 165.86 (C), 165.40 (C), 59.64 (CH), 53.62 (OCH_3-1), 46.57 (CH_2), 29.76 (CH_2) and 25.10 (CH_2) included.

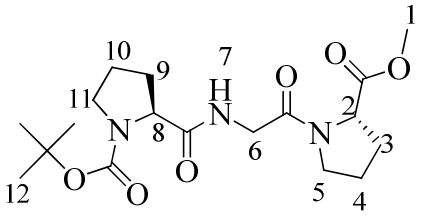
IR (cm^{-1}): 2929 (C-H, br), 1736 (O=C, m), 1655 (O=C, s), 1354 (N-H, C-N, m), 1219 (C-N, N-H, s).

LRMS (ES^+): 187 [$(M + H)^+$, 100%], 373 [$(2M+H)^+$, 3%].

HRMS (ES^+): $C_8H_{15}N_2O_3$ $(M + H)^+$: calculated 187.1077, found 187.1074.

N-Boc-*L*-Pro-Gly-*L*-Pro-OMe 148

Compound **148** was prepared by general procedure 5.2.1 (5.43 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 2% MeOH/CH_2Cl_2) and yielded the title compound as colorless oil (0.90 g, 43%).



^{1}H NMR (400 MHz, d^6-DMSO): δ 7.89 (1NH-7, br); 4.13 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.05 (1H-8, m); 3.91 (2H-6, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.60 and 3.70 (3H-1, 2 x s, there are two singlets from rotamers); 3.55 (2H-5, m); 3.40 (2H-11, m); 2.16 (2H-3, m); 1.93 (2H-10, apparent p, *J* = 8.0 Hz); 1.71-1.84 (2H-9, 2H-4, m); 1.32 and 1.39 (9H-12, 2 x s, there are two singlets from rotamers).

^{13}C NMR (100 MHz, d^6-DMSO): 207.33 (C), 173.25 (C), 167.89 (C), 154.25 (C), 79.37 ($C(CH_3)_3$), 60.47 (CH), 59.37 (CH), 52.67 (OCH_3-1), 47.34 (CH_2), 46.45 (CH_2), 41.82 (CH_2), 31.83 (CH_2), 30.87 (CH_2), 29.48 (CH_2), 28.88 ($C(CH_3)_3$), 25.31 (CH_2). There are extra peaks from rotamers at 173.52 (C), 168.10 (C), 154.58 (C), 60.22 (CH), 58.78 (CH), 53.31 (OCH_3-1), 47.58 (CH_2), 47.08 (CH_2), 31.68 (CH_2), 24.63 (CH_2) and 23.98 (CH_2) included.

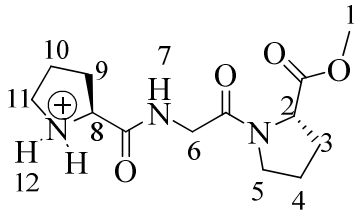
IR (cm^{-1}): 3325 (N-H, br); 2974 (C-H, br), 1743 (O=C, m), 1650 (O=C, s), 1390 (N-H, C-N, s), 1246 (C-N, N-H, s).

LRMS (ES^+): 406 [$(M + Na)^+$, 100%], 789 [$(2M+Na)^+$, 47%].

HRMS (ES^+): $C_{18}H_{29}N_3NaO_6$ $(M + Na)^+$: calculated 406.1949, found 406.1942.

H-*L*-Pro-Gly-*L*-Pro-OMe x HCl 149

Compound **149** was prepared by general procedure 5.2.3 (2.34 mmol). The product was obtained as colorless oil and was used in the next step without further purification (0.63 g, 90%).



^{1}H NMR (400 MHz, d^6-DMSO): δ 10.02 (1NH-12, br); 8.75-8.78 (1NH-7, t, *J* = 6.0 Hz); 8.74 (1NH-12, br); 4.31 (1H-2, dd, *J* = 5.0, 9.0 Hz); 4.24 (1H-8, m); 4.07 (2H-6, 2 x d, *J* = 6.0 Hz, there are two doublets from rotamers); 3.61 and 3.70 (3H-1, 2 x s, there are two singlet from rotamers); 3.52 (2H-5); 3.20 (2H-11); 2.16 (2H-3, m); 1.93 (2H-10, m); 1.71-1.84 (2H-9, 2H-4, m)**.**

^{13}C NMR (100 MHz, d^6-DMSO): 173.20 (C), 169.43 (C), 167.30 (C), 59.13 (CH), 58.93 (CH), 52.31 (OCH_3-1), 46.03 (CH_2), 41.65 (CH_2), 32.80 (CH_2), 30.22 (CH_2), 29.06 (CH_2), 24.87 (CH_2), 23.95 (CH_2).

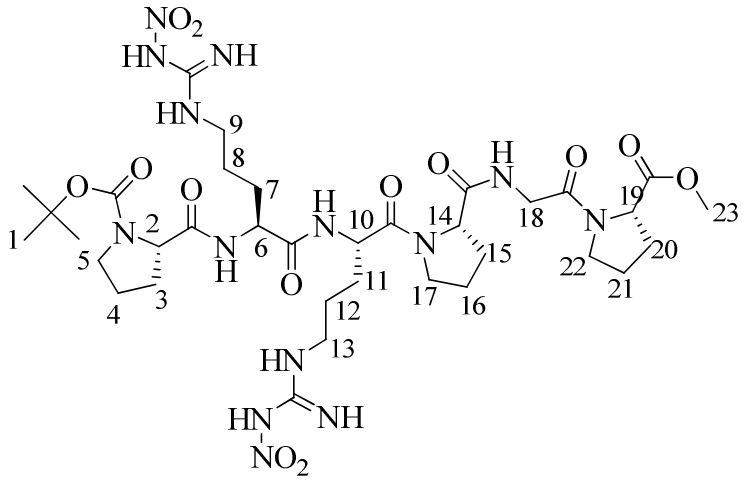
IR (cm^{-1}): 2947 (N-H, br); 2747 (C-H, br), 1738 (O=C, m), 1650 (O=C, s), 1335 (N-H, C-N, m), 1251 (C-N, N-H, s).

LRMS (ES$^+$): 284 [$(M + H)^+$, 100%], 567 [$(2M+H)^+$, 28%].

HRMS (ES$^+$): $C_{13}H_{22}N_3O_4$ $(M + H)^+$: calculated 284.1605, found 284.1604.

***N*-Boc-*L*-Pro-*L*-Arg(NO$_2$)-*L*-Arg(NO$_2$)-*L*-Pro-Gly-*L*-Pro-OMe 150**

Compound **150** was prepared by general procedure 5.2.1 (2.28 mmol). Crude residue was purified by column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) and precipitated by CH_2Cl_2, MeOH and Et_2O and yielded the title compound as a white solid (1.03 g, 51%).



m.p. 78 °C

^{1}H NMR (400 MHz, d^{6}-DMSO): δ 9.72 (1NH, br); 8.52 (1NH, br); 8.36 (1NH, d, *J* =12.0 Hz), 8.29 (1NH, d, *J* = 8.0 Hz); 8.19 (1NH, d, *J* = 8.0 Hz); 7.91 (1NH, d, *J* = 8.0 Hz); 3.87-4.59 (1H-2, 1H-6, 1H-10, 1H-14, 2H-18, 1H-19, m); 3.60 and 3.61 (3H-23, s, there are two singlets from rotamers); 3.10-3.71 (2H-5, 2H-9, 2H-13, 2H-17, 2H-22, m); 1.57-2.09 (2H-3, 2H-4, 2H-11, 2H-12, 2H-15, 2H-16, 2H-20, 2H-21); 1.31 and 1.38 (9H-1, 2 x s, there are two singlets from rotamers). There are 3 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (100 MHz, d^{4}-MeOD): 175.55 (C), 174.32 (C), 174.16 (C), 173.79 (C), 172.32 (C), 169.11 (C), 160.80 (C), 156.38 (C), 155.86 (C), 81.36 ($C(CH_3)_3$), 61.62 (CH), 61.34 (CH), 60.44 (CH), 54.13 (CH), 52.97 (OCH_3-23), 52.17 (CH), 48.74 (CH_2), 48.39 (CH_2), 47.99 (CH_2), 47.43 (CH_2), 42.74 (CH_2), 41.82 (CH_2), 32.49 (CH_2), 31.39 (CH_2), 30.61 (CH_2), 30.06 (CH_2), 29.54 (CH_2), 28.83 ($C(CH_3)_3$), 25.99 (CH_2), 25.73 (CH_2), 25.51 (CH_2), 24.71 (CH_2), 23.24 (CH_2). There are extra peaks from rotamers at 175.30 (C), 173.64 (C), 169.48 (C), 169.21 (C), 160.86 (C), 81.27 ($C(CH_3)_3$), 62.09 (CH), 60.05 (CH), 55.09 (CH), 53.47 (OCH_3-23), 37.56 (CH_2), 36.37 (CH_2), 29.70 ($C(CH_3)_3$) and 23.45 (CH_2) included.

IR (cm^{-1}): 3307 (N-H, br); 2953 (C-H, br), 1741 (O=C, m), 1634 (O=C, s), 1366 (N-H, C-N, s), 1258 (C-N, N-H, s).

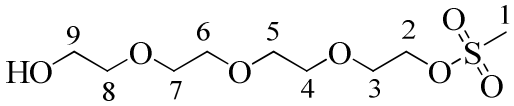
LRMS (ES^{+}): 905 [$(M + Na)^{+}$, 50%].

HRMS (ES^{+}): $C_{35}H_{58}N_{14}NaO_{13}$ $(M + Na)^{+}$: calculated 905.4200, found 905.4180.

5.5.2) Synthesis of polyethyleneglycol tether (PEG tether): *N*-Boc protection of PEG linked amino alcohol 155.

Synthesis of *mono*-mesylation of tetraethylene glycols (Methanesulfonic acid 2-[2-[2-(2-hydroxyethoxyethoxy]ethoxy]ethyl ester) 151.

To a stirred solution of tetraethylene glycol (20.0 mmol, 0.65 M) in THF was slowly added triethylamine (1.0 equiv, 20.0 mmol) and methylsulphonyl chloride (1.0 equiv, 20.0 mmol), respectively at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and stirred overnight at room temperature. Precipitate was removed by suction filtration and washed with diethyl ether (15 mL required for a 20 mmol scale preparation). All solvents were removed under reduced pressure and crude residue was purified by column chromatography (20% EtOAc/hexane to 100% EtOAc) to obtain the title compound **151** as colorless oil (1.14 g, 21%).

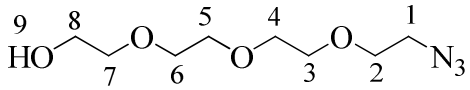


^{1}H NMR (400 MHz, $CDCl_3$): δ 4.32 (2H-2, t, *J* = 4.0 Hz); 3.53-3.73 (2H-3, 2H-4, 2H-5, 2H-6, 2H-7, 2H-8, 2H-9, m); 3.01 (3H-1, s).
^{13}C NMR (100 MHz, $CDCl_3$): δ 71.48 (CH_2), 69.61 (CH_2), 69.59 (CH_2), 69.45 (CH_2), 69.29 (CH_2), 68.19 (CH_2), 68.00 (CH_2), 60.69 (CH_2), 36.64 (CH_3).
IR (cm^{-1}): 3442 (O-H, br), 2873 (C-H, br), 1347 (S=O, s), 1172 (C-O-C, s), 974 (S-O-C, s).
LRMS (ES^{+}): 295 [$(M + Na)^{+}$, 100%], 567 [$(2M+Na)^{+}$, 10%].
HRMS (ES^{+}): $C_9H_{20}NaO_7S$ $(M + Na)^{+}$: calculated 295.0822, found 295.0819.

Preparation of PEG-linked azido alcohols 12 (2-[2-[2-(2-Azidoethoxy) ethoxy]ethoxy] ethanol) 152.

To a stirred solution of *mono*-mesylated compound **151** (4.2 mmol, 0.57 M) in MeCN was added sodium azide (1.5 equiv, 6.3 mmol) and heated under reflux for 36 h. The reaction mixture was cooled down to room temperature and added H_2O (25 mL required for a 4.2 mmol scale preparation). The mixture was extracted with CH_2Cl_2 (120 mL required for a 4.2 mmol scale preparation) and dried over $MgSO_4$ and concentrated *in vacuo.* The crude

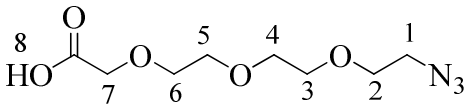
residue was purified on silica gel column chromatography (20% EtOAc/hexane) to obtain the title product **152** as colorless oil (0.81 g, 88%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 3.61 (2H-8, t, *J* = 4.0 Hz); 3.57-3.59 (2H-3, 2H-4, 2H-5, 2H-6, 2H-7, m); 3.50 (2H-2, t, *J* =4.0 Hz); 3.30 (2H-1, t, *J* = 4.0 Hz); 3.11 (OH-9, br).
^{13}C NMR (100 MHz, $CDCl_3$): δ 72.26 (CH_2), 70.32 (CH_2), 70.27 (CH_2), 70.20 (CH_2), 69.96 (CH_2), 69.66 (CH_2), 61.24 (CH_2), 50.32 (CH_2).
IR (cm^{-1}): 3416 (O-H, br), 2869 (C-H, br), 2098 (N_3, s), 1100 (C-O-C, s).
LRMS (ES$^+$): 242 [$(M + Na)^+$, 100%]
HRMS (ES$^+$): $C_8H_{17}N_3NaO_4$ $(M + Na)^+$: calculated 242.1111, found 242.1111.

Jones' oxidation of PEG-linked azido alcohol 153.

Jones' reagent (2.67 M solution) was freshly prepared from 2.67 g CrO_3 and 2.3 mL conc. H_2SO_4 and made up final volume with water to 10.0 mL.
To a stirred solution of PEG-linked azido alcohol **152** (13 mmol) in acetone was added Jones' reagent (8.3 mL of 2.67 M solution) dropwise. The reaction mixture was stirred at room temperature for 1 h and then EtOH (2 mL for 13 mmol preparation) was added followed by water (10 mL for 13 mmol preparation) to dissolve the chromium salts precipitated. The reaction mixture was extracted with CH_2Cl_2 (3 x 20 mL). The combined CH_2Cl_2 extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The oily residue was taken into CH_2Cl_2 (30 mL for a 13 mmol preparation) and basified by addition of KOH (1M aqueous solution) before extraction with H_2O (20 mL required for a 13 mmol preparation). The aqueous layer was separated and acidified with HCl (1M aqueous solution), then re-extracted again with CH_2Cl_2 (4 x 10 mL required for a 13 mmol preparation). The organic extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The titled compound **153** was obtained as colorless oil (1.90 g, 63%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 9.62 (OH-8, br); 4.16 (2H-7, s); 3.64-3.75 (2H-2, 2H-3, 2H-4, 2H-5, 2H-6, m); 3.38 (2H-1, t, *J* = 4.0 Hz).

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.75 (C), 71.04 (CH_2), 70.49 (CH_2), 70.35 (CH_2), 70.27 (CH_2), 69.89 (CH_2), 68.37 (CH_2), 50.51 (CH_2).

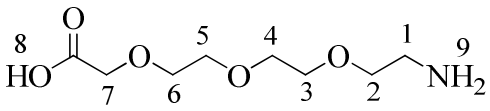
IR (cm^{-1}): 2873 (O-H, br), 2099 (N_3, s), 1736 (C=O, s), 1112 (C-O-C, s).

LRMS (ES$^+$): 256 [$(M + Na)^+$, 100%].

HRMS (ES$^+$): $C_8H_{15}N_3NaO_5$ $(M + Na)^+$: calculated 256.0904, found 256.0902.

PEG-linked azides reduction 154.

To a stirred solution of the azido acid **153** (3.1 mmol, 0.6 M) in MeOH was added triethylamine (6.0 equiv, 18.6 mmol) and 1,3-propanedithiol (5.0 equiv, 15.5 mmol), respectively at room temperature. The reaction mixture was stirred for 3 days and then all solvents were removed *in vacuo*. The crude residue was diluted with water (50 mL required for a 3.1 mmol preparation) and extracted with diethyl ether (50 mL required for 3.1 mmol preparation) to remove excess reducing agent. The aqueous layer was collected and concentrated *in vacuo*. The title product **154** was obtained as pale yellow oil which was used in the next step without further purification.



^{1}H NMR (400 MHz, $CDCl_3$): δ 6.54 (OH-8, br); 3.92 (2H-7, s); 3.60-3.79 (2H-2, 2H-3, 2H-4, 2H-5, 2H-6, m); 3.15 (2H-1, m). There are 1 x NH_2 protons missing from ^{1}H-spectrum.

^{13}C NMR (150 MHz, $CDCl_3$): δ 175.78 (C), 71.15 (CH_2), 70.21 (CH_2), 70.03 (CH_2), 69.85 (CH_2), 69.33 (CH_2), 67.60 (CH_2), 39.05 (CH_2). There are extra peaks from impurities at 72.60 (CH_2), 69.95 (CH_2), 67.76 (CH_2) and 39.58 (CH_2) included.

IR (cm^{-1}): 3500 (N-H, br), 2874 (O-H, br), 1577 (C=O and N-H, br), 1089 (C-O-C, s).

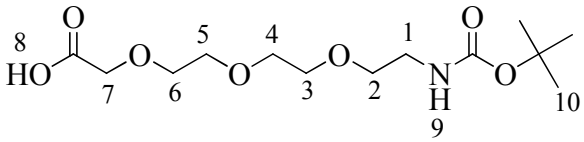
LRMS (ES$^+$): 208 [$(M+H)^+$, 60%], 415 [$(2M+H)^+$, 20%], 644 [$(3M+H)^+$, 5%].

HRMS (ES$^+$): $C_8H_{18}N_3O_5$ $(M + H)^+$: calculated 208.1179, found 208.1183.

***N*-Boc protection of PEG-linked tether 155.**

To a stirred solution of compound **154** (3.0 mmol) in H_2O (3 mL) was added potassium hydroxide (KOH) (6 mL of a 1M aqueous solution, 6.0 mmol) and di-*tert*-butyldicarbonate (1.2 equiv, 3.6 mmol) in THF (3 mL) at room temperature. The reaction mixture was stirred overnight and then adjusted to pH 5 by addition of HCl (1 M aqueous solution). The

reaction mixture was extracted with CH_2Cl_2 (2 x 50 mL), organic phases were combined, dried over $MgSO_4$ and concentrated *in vacuo*. The crude residue was purified by column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2). The title product **155** was obtained as colorless oil (0.75 g, 81%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 5.23 (OH-8, br); 4.17 (2H-7, s); 3.77 (2H-2, t, *J* = 4.0 Hz); 3.54-3.72 (2H-3, 2H-4, 2H-5, 2H-6, m); 3.31 (2H-1, t, *J* = 4.0 Hz); 1.45 (9H-10, s). There is one N-H proton missing from ^{1}H-spectrum.

^{13}C NMR (150 MHz, $CDCl_3$): δ 172.21 (C), 156.37 (C), 79.59 ($C(CH_3)_3$), 71.34 (CH_2), 70.51 (CH_2), 70.23 (CH_2), 70.00 (CH_2), 68.80 (CH_2), 58.40 (CH_2), 40.57 (CH_2), 28.37 ($C(CH_3)_3$).

IR (cm^{-1}): 3351 (N-H, br), 2872 (C-H, br), 1690 (C=O, s), 1366 (N-H, C-N, m), 1249 (C-N, N-H, m), 1098 (C-O-C, s).

LRMS (ES$^-$): 306 [$(M-H)^-$, 100%].

HRMS (ES$^+$): $C_{13}H_{25}NNaO_7$ $(M + Na)^+$: calculated 330.1523, found 330.1529.

5.5.3) General procedure for cyclic peptide synthesis.

General procedure A: coupling reaction of *N*-Boc protection of PEG-linked tether and free N-terminus peptide

To a stirred solution of *N*-Boc-PEG-linked tether **155** (0.6 mmol) in DMF (5 mL) was added HOBt (2 equiv, 1.2 mmol) followed by DCC (1 equiv, 0.6 mmol). The mixture was stirred at room temperature for 15 min before cooling to 0 °C and addition of a solution of free *N*-terminus hexapeptide (1 equiv, 0.6 mmol) and DIPEA (10 equiv, 6.0 mmol) in DMF (5 mL) dropwise over 15 min. The reaction mixture was stirred at 0 °C for 1 h before warming to room temperature and stirring overnight. The mixture was filtered, washed with DMF (20 mL for 0.6 mmol preparation) and concentrated *in vacuo*. Crude residue was purified by column chromatography.

General procedure B: ester hydrolysis

To a stirred solution of *N*-PEG-*N*'-Boc peptide ester (0.24 mmol) in H_2O (2.0 mL) was added 1 M NaOH (1.5 equiv, 0.36 mmol) and stirred at room temperature for 3 h. The completely reaction was monitored by Mass spectroscopy. All solvents were removed and concentrated *in vacuo*. The desired product was obtained as pale yellow oil and could be used in the next step without further purification

General procedure C: pentafluorophenol protection at *C*-terminus of peptide

To a stirred solution of crude *N*-PEG-*N'*-Boc-hexapeptide-ONa (0.25 mmol), Pfp-OH (2 equiv, 0.50 mmol) and DMAP (1 mg) in DMF (2 mL) at 0 °C was added EDC (2 equiv, 0.50 mmol) and the mixture stirred at 0 °C for 30 min before warming to room temperature and stirring for 12 h. The mixture was then concentrated *in vacuo*. The resulting oil was taken into $CHCl_3$ (75 mL for 0.26 mmol preparation) and washed with HCl (5 mL of a 1 M aqueous solution, for 0.26 mmol preparation) before concentration *in vacuo* and once more. The crude product could be used in the next step without further purification.

General procedure D: deprotection of *N*-Boc group

The *N*-Boc peptide (about 0.24 mmol) was treated with 4 M HCl in dioxane (3 equiv, 0.72 mmol) at 0 ºC. The solution was stirred at room temperature for 1 h. All solvents were removed and concentrated *in vacuo*. The crude product could be used in the next step without further purification.

General procedure E: *N,C*-PEG-tethered-hexapeptide cyclization

To a vigorously stirred solution of 0.21 mL of DIPEA in MeCN (70 mL, 0.003 M) was added crude *N*-PEG-hexapeptide-Opfp salt (about 0.23 mmol) in MeCN (24 mL, for 0.23 mmol preparation) via syringe pump at 70 °C over 6 h. The mixture was stirred at 70 °C for further 2 h before cooling. The reaction mixture was stirred at room temperature overnight and concentrated *in vacuo*. The crude residue was purified by column chromatography.

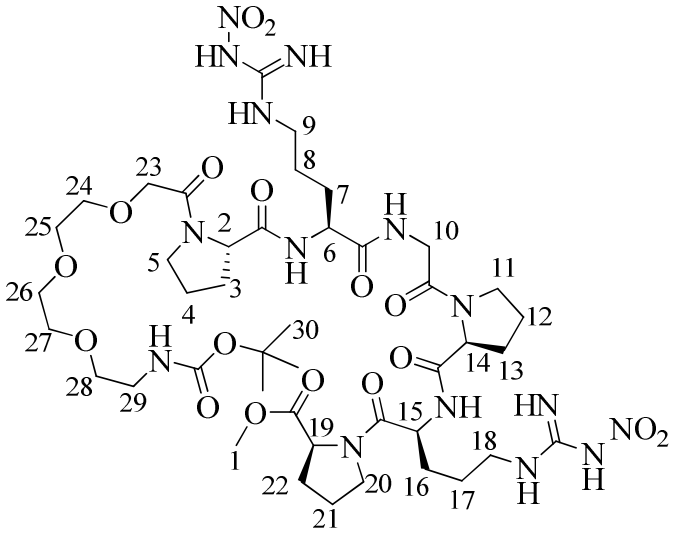
General procedure F: catalytic hydrogenation

To a stirred solution of cyclic peptide (0.028 mmol) in MeOH: AcOH (4 mL of a 3: 1 mixture) was added Pd/C (2 mg) and reaction mixture was stirred under H_2 (1 atm) at room temperature for 17 h. The mixture was then filtered through celite^{TM}, the filter cake was washed with MeOH (20 mL for 0.028 mmol preparation) and the filtrate was concentrated *in vacuo*.

5.5.4) Synthesis of poly(ethylene glycol) tethered cyclic peptide (*N*,C-PEG-tethered-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro x AcOH salt) 161.

N-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro OMe 156.

Compound **156** was prepared from H-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe x HCl, **69** (0.6 mmol) by general procedure A. Crude residue was purified by column chromatography (CH_2Cl_2: MeOH (9:1) to (8.5: 1.5)) and gave an oily solid which was washed with EtOAc (20 mL). The title compound was obtained as a pale yellow solid (0.26 g, 40%).



m.p. 72 °C

^{1}H NMR (400 MHz, DMSO, 100 °C): δ 8.51 (1NH, br); 8.41 (1NH, d, *J* = 4.0 Hz); 8.16 (1NH, d, *J* = 8.0 Hz); 8.12 (1NH, d, *J* = 8.0 Hz); 8.07 (1NH, d, *J* = 8.0 Hz); 8.00 (1NH, d, *J* = 4.0 Hz); 7.89 (1NH, br); 6.74 (1NH, br); 4.11-4.53 (1H-2, 1H-6, 2H-10, 1H-14, 1H-15, 1H-19, m); 3.60 (3H-1, s); 3.05-4.05 (2H-5, 2H-9, 2H-11, 2H-18, 2H-22, 2H-23, 2H-24, 2H-25, 2H-26, 2H-27, 2H-28, 2H-29, m); 1.54-2.21 (2H-3, 2H-4, 2H-7, 2H-8, 2H-12, 2H-

13, 2H-16, 2H-17, 2H-21, 2H-22, m); 1.37 (9H-30, s). There are 2 x N-H protons missing from ^{1}H-spectrum.

^{13}C NMR (150 MHz, MeOD): δ 174.98 (C), 174.72 (C), 174.65 (C), 174.45 (C), 174.24 (C), 172.48 (C), 171.54 (C), 169.86 (C), 161.29 (C), 158.79 (C), 80.48 ($C(CH_3)_3$), 72.13 (CH_2), 71.89 (CH_2), 71.67 (CH_2), 71.55 (CH_2), 71.12 (CH_2), 62.30 (CH), 61.93 (CH), 60.91 (CH), 55.46 (CH), 53.91 (CH), 52.23 (OCH_3), 49.84 (CH_2), 48.84 (CH_2), 48.32 (CH_2), 48.01 (CH_2), 44.26 (CH_2), 43.30 (CH_2), 42.32 (CH_2), 41.71 (CH_2), 33.92 (CH_2), 33.69 (CH_2), 32.63 (CH_2), 30.99 (CH_2), 30.89 (CH_2), 30.50 (CH_2), 29.97 (CH_2), 29.39 ($C(CH_3)_3$), 26.52 (CH_2), 26.44 (CH_2), 26.29 (CH_2). There are extra peaks from rotamers at 169.73 (C), 61.30 (CH) and 52.63 (CH) included.

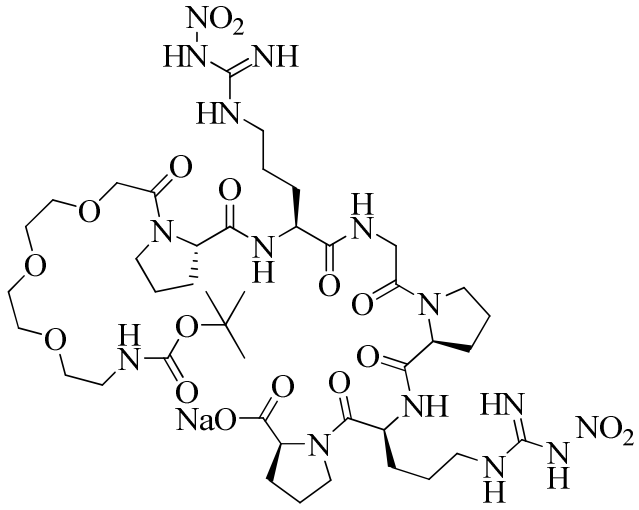
IR (cm^{-1}): 3239 (N-H, br), 2952 (C-H, br), 1741 (C=O, m), 1622 (C=O, s), 1531 (N=O, m), 1365 (N-H, C-N, s), 1250 (C-N, N-H, s).

LRMS (ES^{+}): 558 [$(M+2Na)^{2+}$, 33%], 1094[$(M + Na)^{+}$, 30%].

HRMS (ES^{+}): accurate mass could not detect on compound which more than 1000 Da.

_N_-PEG-_N'_- Boc-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro-ONa 157.

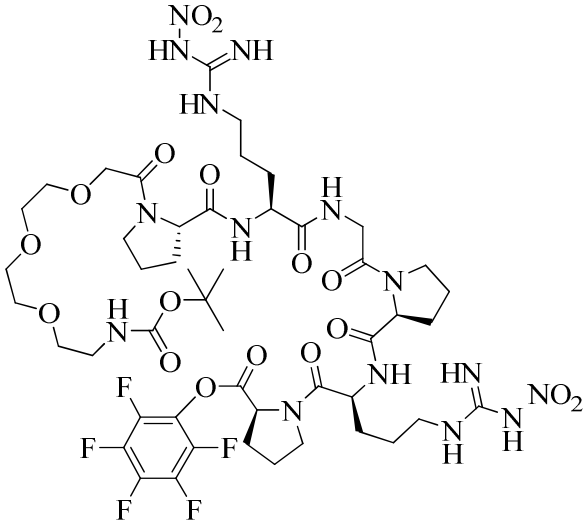
Compound **157** was prepared from hexapeptide **156**, _N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro-OMe, by general procedure B. The desired product **157** was obtained as pale yellow oil and could be used in the next step without further purification (0.25 g, 95%).



LRMS (ES^{+}): 541 [$(M+2H)^{2+}$, 9%], 552 [$(M+Na+H)^{2+}$, 5%], 563 [$(M+2Na)^{2+}$, 100%], 571 [$(M+Na+NH4)^{2+}$, 1%], 1102 [$(M+Na)^{+}$, 38%].

_N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro-Opfp 158.

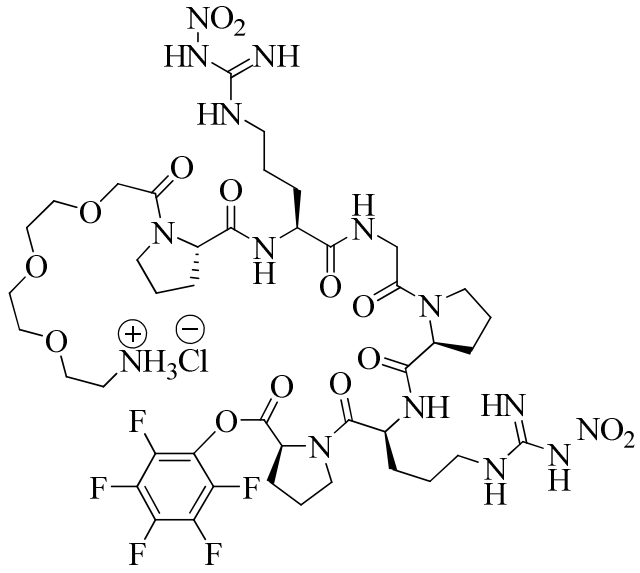
Compound **158** was prepared from hexapeptide **157**, _N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro-ONa, by general procedure C. The desired product **158** was obtained as pale yellow oil and could be used in the next step without further purification (0.26 g, 93%).



LRMS (ES$^+$): 632 [$(M+Na+NH_4)^{2+}$, 10%], 1246 [$(M+Na)^+$, 4%].

_N_-PEG-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro-Opfp x HCl 159.

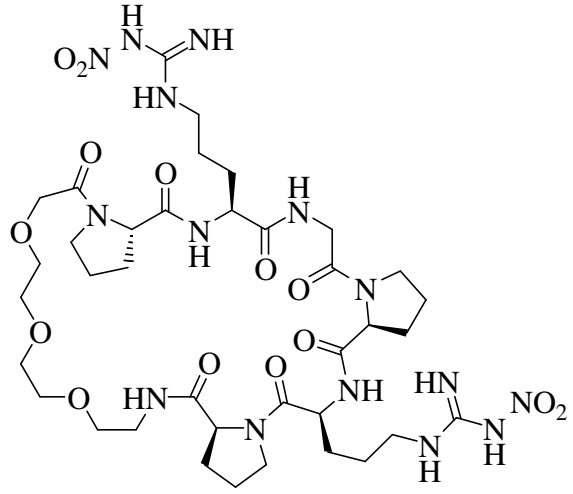
Compound **159** was prepared from crude pfp-ester **158**, _N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro-Opfp, by general procedure D. The desired product **159** was obtained as pale yellow oil and could be used in the next step without further purification (0.24 g, 98%).



LRMS (ES$^+$): 574 [(M+H+Na)$^{2+}$, 20%], 581 [(M+Na+NH$_4$)$^{2+}$, 7%], 584 [(M+2Na)$^{2+}$, 2%], 1124 [(M+H)$^+$, 15%].

_N_,C-PEG-tethered-_L_-Pro-_L_-Arg(NO$_2$)-Gly-_L_-Pro-_L_-Arg(NO$_2$)-_L_-Pro 160.

Compound **160** was prepared from hexapeptide **159**, _N_-PEG-_L_-Pro-_L_-Arg(NO$_2$)-Gly-_L_-Pro-_L_-Arg(NO$_2$)-_L_-Pro-Opfp x HCl, by general procedure E. Crude residue was purified by precipitation from MeCN with Et$_2$O, followed by column chromatography (SiO$_2$ eluted with CHCl$_3$: MeOH: H$_2$O (65: 25: 1 to 65: 25: 4), precipitation once more from MeCN with Et$_2$O and washing with Et$_2$O (5 mL) gave the title compound **160** as a white solid (19 mg, 10%).

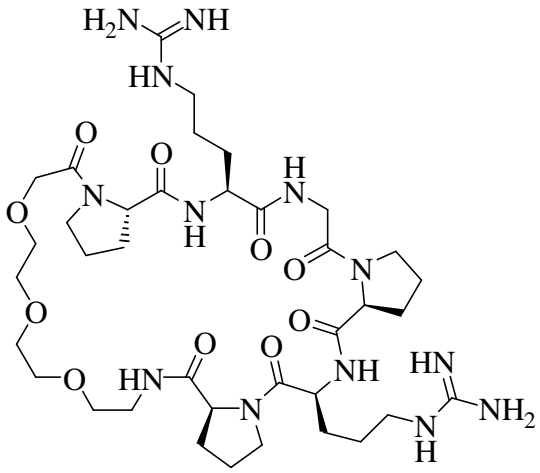


LRMS (ES$^+$): 493 [(M+2Na)$^{2+}$, 100%], 962 [(M+Na)$^+$, 50%].

HRMS (ES$^+$): $C_{37}H_{61}N_{15}NaO_{14}$ (M+ Na)$^+$: calculated 962.4415, found 962.4408.

_N_,C-PEG-tethered-_L_-Pro-_L_-Arg(H)-Gly-_L_-Pro-_L_-Arg(H)-_L_-Pro x AcOH salt 161.

Compound **161** was prepared from _N_,C-PEG-tethered-_L_-Pro-_L_-Arg(NO_2)-Gly-_L_-Pro-_L_-Arg(NO_2)-_L_-Pro **160** by general procedure F. Crude residue was triturated with Et_2O to obtain the title compound **161** as a white solid (12 mg, 72%).

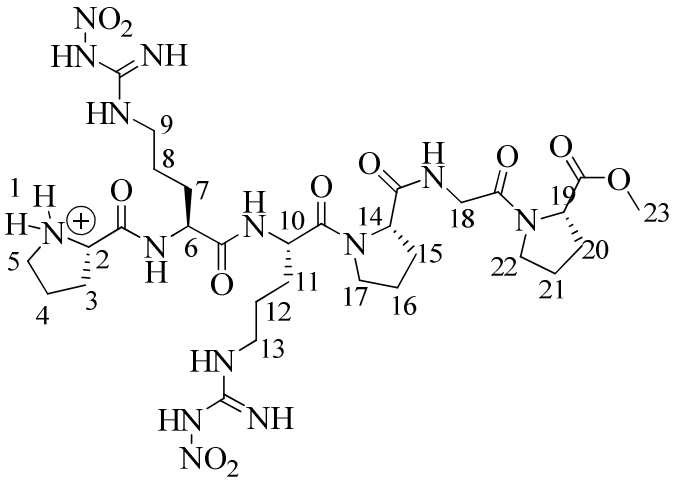


LRMS (ES$^+$): 179 [$(M+2Na+3H)^{5+}$, 21%], 193 [$(M+5Na)^{5+}$, 12%], 219 [$(M+Na+3H)^{4+}$, 68%].
LCMS (ES$^+$): 425.7 [$(M+2H)^{2+}$].

5.5.5) Synthesis of poly(ethylene glycol) tethered cyclic peptide (_N_,C-PEG-tethered-_L_-Pro-_L_-Arg(H)-_L_-Arg(H)-_L_-Pro-Gly-_L_-Pro x AcOH salt) 168.

H-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-OMe x HCl 162.

Compound **162** was prepared from _N_-Boc-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-OMe, **150** (0.6 mmol) by general procedure D. The crude residue was precipitated by MeOH and diethyl ether to obtained title compound as a white solid and could be used in the next step without further purification (0.47 g, 95%).



m.p. 105 °C

^{1}H NMR (400 MHz, DMSO): δ 9.46 (1NH, br); 8.67 (1NH, d, *J* = 8.0 Hz); 8.48 (1NH, br); 8.18 (1NH, d, *J* = 8.0 Hz); 7.87-7.96 (6NH, br); 4.20-4.74 (1H-2, 1H-6, 1H-10, 1H-14, 2H-18, 1H-19, m); 3.17-3.69 (2H-5, 2H-9, 2H-13, 2H-17, 2H-22, m); 3.61 (3H-23, s); 1.53-2.27 (2H-3, 2H-4, 2H-11, 2H-12, 2H-15, 2H-16, 2H-20, 2H-21, m).

^{13}C NMR (150 MHz, MeOD): δ 175.64 (C), 173.68 (C), 173.45 (C), 173.42 (C), 171.65 (C), 170.15 (C), 169.30 (C), 160.66 (C), 61.17 (CH), 60.61 (CH), 58.79 (CH), 54.94 (CH), 52.17 (OCH_3), 49.43 (CH), 49.16 (CH_2), 48.95 (CH_2), 48.91 (CH_2), 48.86 (CH_2), 47.82 (CH_2), 47.57 (CH_2), 42.14 (CH_2), 31.32 (CH_2), 30.31 (CH_2), 30.18 (CH_2), 29.79 (CH_2), 29.47 (CH_2), 26.14 (CH_2), 26.07 (CH_2), 25.82 (CH_2), 25.23 (CH_2). There are extra peaks from rotamers at 61.80 (CH), 54.89 (CH), 49.64 (CH_2), 49.43 (CH_2), 49.22 (CH_2), 49.14 (CH_2) included.

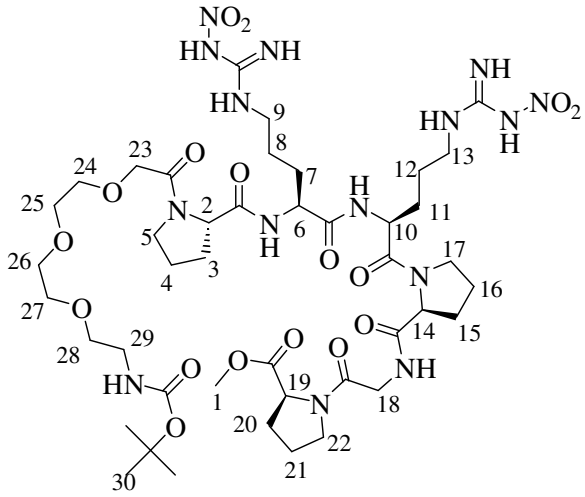
IR (cm^{-1}): 3274 (N-H, br), 2956 (C-H, br), 1741 (C=O, m), 1626 (C=O, s), 1530 (N=O, m), 1266 (C-N, N-H, s).

LRMS (ES^{+}): 403 [$(M+H+Na)^{2+}$, 53%], 783 [$(M + H)^{+}$, 100%].

HRMS (ES^{+}): $C_{30}H_{51}N_{14}O_{11}$ $(M+ H)^{+}$: calculated 783.3856, found 783.3849.

***N*-PEG-*N*'-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe 163**.

Compound **163** was prepared from hexapeptide **162**, H-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe x HCl, (0.6 mmol) by general procedure A. Crude residue was purified by column chromatography (CH_2Cl_2: MeOH (9:1) to (8.5: 1.5)) and gave an oily solid which was washed with EtOAc (20 mL). The title compound **163** was obtained as a pale yellow solid (0.26 g, 42%).



m.p. 76 °C

^{1}H NMR (400 MHz, DMSO, 80 °C): δ 8.51 (1NH, br); 8.09 (1NH, d, *J* = 8.0 Hz); 8.01 (1NH, d, *J* = 8.0 Hz); 7.95 (1NH, t, *J* = 8.0 Hz); 7.96 (1NH, d, *J* = 8.0 Hz); 7.89-7.94 (4NH, br); 6.74 (1NH, t, *J* = 4.0 Hz); 4.00-4.75 (1H-2, 1H-6, 1H-10, 1H-14, 2H-18, 1H-19, m); 3.61 and 3.69 (3H-1, s, there are two singlets from rotamers); 3.02-3.80 (2H-5, 2H-9, 2H-13, 2H-17, 2H-22, 2H-23, 2H-24, 2H-25, 2H-26, 2H-27, 2H-28, 2H-29, m); 1.50-2.16 (2H-3, 2H-4, 2H-7, 2H-8, 2H-11, 2H-12, 2H-15, 2H-16, 2H-20, 2H-21, m); 1.37 (9H-30, s).

^{13}C NMR (150 MHz, MeOD): δ 174.67 (C), 174.56 (C), 174.35 (C), 173.75 (C), 172.46 (C), 171.25 (C), 169.26 (C), 161.03 (C), 161.01 (C), 158.61 (C), 80.22 ($C(CH_3)_3$), 71.72 (CH_2), 71.50 (CH_2), 71.43 (CH_2), 71.32 (CH_2), 71.30 (CH_2), 70.78 (CH_2), 70.70 (CH_2), 61.88 (CH), 60.57 (CH), 60.26 (CH), 54.16 (CH), 53.01 (CH), 52.25 (OCH_3), 48.87 (CH_2), 48.47 (CH_2), 47.62 (CH_2), 42.82 (CH_2), 42.05 (CH_2), 41.88 (CH_2), 41.34 (CH_2), 33.59 (CH_2), 33.12 (CH_2), 32.25 (CH_2), 30.69 (CH_2), 30.60 (CH_2), 30.16 (CH_2), 28.93 ($C(CH_3)_3$), 26.13 (CH_2), 25.82 (CH_2), 23.32 (CH_2). There are extra peaks from rotamers at 62.20 (CH), 61.75 (CH), 52.34 (OCH_3), 40.16 (CH_2), 37.62 (CH_2), 36.44 (CH_2) and 29.60 (CH_2) included.

IR (cm^{-1}): 2954 (C-H, br), 1740 (C=O, m), 1674 (C=O, s), 1540 (N=O, m), 1360 (N-H, C-N, m), 1266 (C-N, N-H, s).

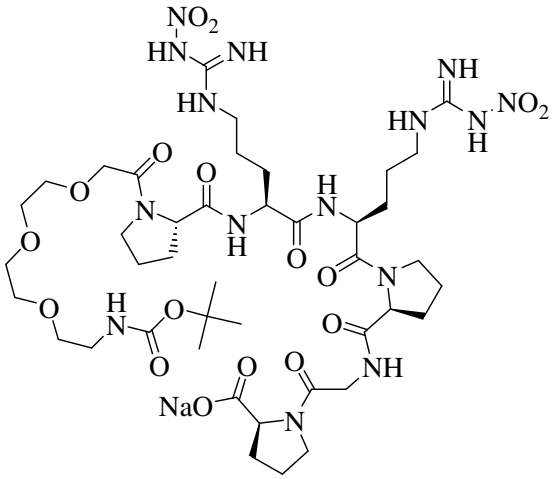
LRMS (ES^{+}): 559 [$(M+2Na)^{2+}$, 10%], 1094 [$(M + Na)^{+}$, 4%].

HRMS (ES^{+}): accurate mass could not detect on compound which more than 1000 Da.

LCMS (ES^{+}): 486.6 [$(M\text{-Boc})+2H)^{2+}$].

_N_-PEG-_N'_- Boc-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-ONa 164.

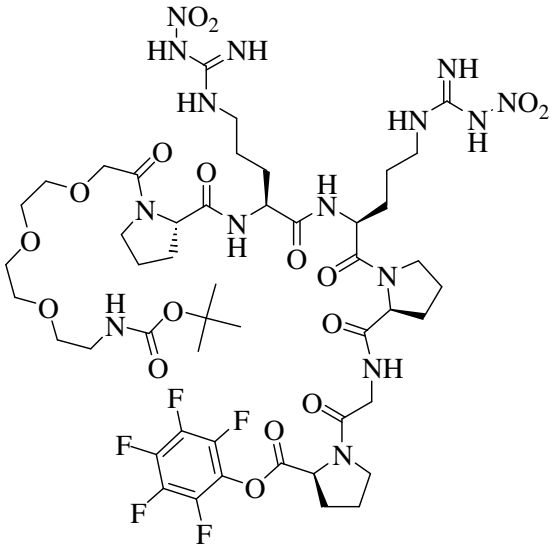
Compound **164** was prepared from hexapeptide **163**, _N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-OMe, by general procedure B. The desired product **164** was obtained as pale yellow oil and could be used in the next step without further purification (0.27 g, 98%).



LRMS (ES$^+$): 541 [$(M+2H)^{2+}$, 2%], 563 [$(M+2Na)^{2+}$, 32%], 1102 [$(M+Na)^+$, 9%].

_N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-Opfp 165.

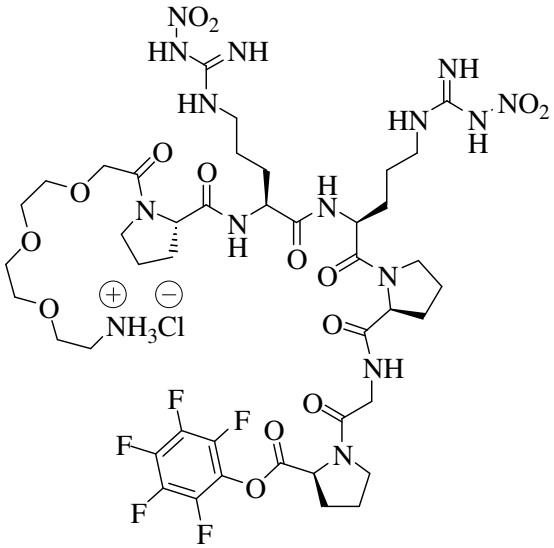
Compound **165** was prepared from hexapeptide **164**, _N_-PEG-_N'_- Boc-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-ONa, by general procedure C. The desired product **165** was obtained as pale yellow oil and could be used in the next step without further purification (0.29 g, 95%).



LRMS (ES$^+$): 613 [(M+2H)$^{2+}$, 4%], 623 [(M+H+Na)$^{2+}$, 2%], 635 [(M+2Na)$^{2+}$, 54%], 1246 [(M+Na)$^+$, 35%].

_N_-PEG-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-Opfp x HCl 166.

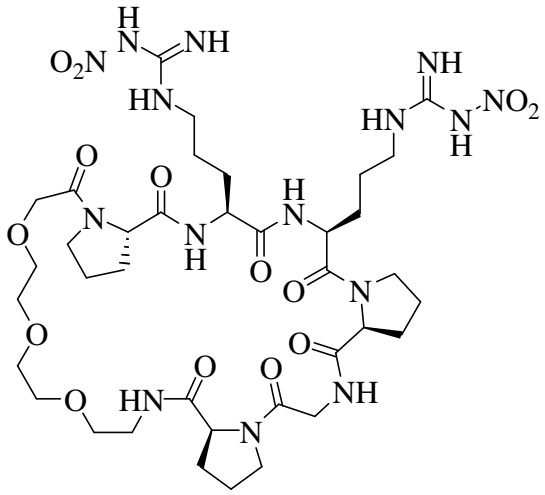
Compound **166** was prepared from crude pfp-ester **165**, _N_-PEG-_N'_-Boc-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro-Opfp, by general procedure D. The desired product **166** was obtained as pale yellow oil and could be used in the next step without further purification (0.26 g, 97%).



LRMS (ES$^+$): 563 [(M+2H)$^{2+}$, 5%], 574 [(M+H+Na)$^{2+}$, 100%], 581 [(M+Na+NH_4)$^{2+}$, 10%], 585 [(M+2Na)$^{2+}$, 6%], 1124 [(M+H)$^+$, 30%], 1146 [(M+Na)$^+$, 4%].

_N_,C-PEG-tethered-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro 167.

Compound **167** was prepared from hexapeptide **166**, _N_,C-PEG-tethered-_L_-Pro-_L_-Arg(NO_2)-_L_-Arg(NO_2)-_L_-Pro-Gly-_L_-Pro, by general procedure E. Crude residue was purified by precipitation from MeCN with Et_2O, followed by column chromatography (SiO_2 eluted with $CHCl_3$: MeOH: H_2O (65: 25: 1 to 65: 25: 4), precipitation once more from MeCN with Et_2O and washing with Et_2O (5 mL) gave the title compound **98** as a white solid (26 mg, 12%).

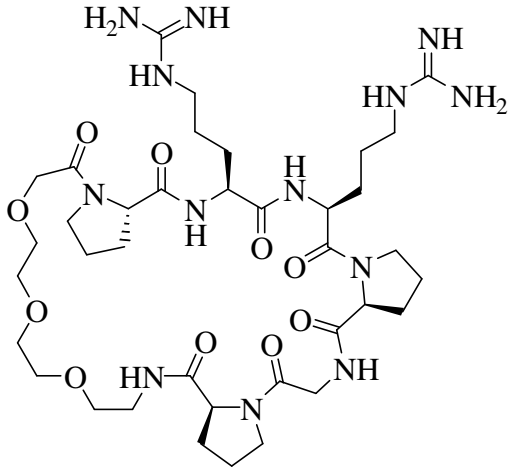


LRMS (ES$^+$): 492 [(M+2Na)$^{2+}$, 80%], 962 [(M+Na)+, 40%].

HRMS (ES$^+$): $C_{37}H_{61}N_{15}NaO_{14}$ (M+ Na)$^+$: calculated 962.4415, found 962.4439

***N*,C-PEG-tethered-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro x AcOH salt 168.**

Compound **168** was prepared from *N*,C-PEG-tethered-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro **167** by general procedure F. Crude residue was triturated with Et_2O to obtain the title compound **168** as a white solid (9 mg, 70%).



LRMS (ES$^+$): 179 [(M+2Na+3H)$^{5+}$, 21%], 193 [(M+5Na)$^{5+}$, 5%], 219 [(M+Na+3H)$^{4+}$, 100%].

LCMS (ES$^+$): 425.5 [(M+2H)$^{2+}$].

CHAPTER 6: MATERIALS AND METHODS

6.1 GENERAL TECHNIQUE MATERIALS AND CULTURE CONDITIONS

All media preparation and other cell culture work were performed in a laminar flow hood. 70% Ethanol was used to clean all surfaces. Sterile pipettes, disposable test tube and autoclaved pipette tips were used for cell culture. All culture vessels, test tubes, pipette tip boxes were opened only in the laminar flow hood. Cells were cultured at 37 °C in a humidified atmosphere containing 5 or 10% CO_2 depending on the cell line.

6.1.1) Materials

From Greiner UK: all tissue cultured plastic ware

From Invitrogen life technologies UK:

Hank's Balanced Salt Solution (HBSS)	Cat. No. 14170088
Dulbecco's Modified Eagle Medium (DMEM)	Cat. No. 41966029

From BioWhittaker:

Nutrient Mixture F-12 Medium (Ham's F-12)	Cat. No. BE12-615F

From LONZA:

Trypsin-EDTA	Cat. No. BE17-161E
Penicillin-Streptomycin	Cat. No. DE17-603E
[5,000 units/ml Potassium Penicillin and 5,000 mcg/ml Streptomycin Sulfate]	
L-Glutamine	Cat. No. BE17-605E
[200 mM solution in 0.85% NaCl]	

From Laboratory stock solution:

Phosphate Buffered Saline (PBS) pH 7.2

(30X) 600 g NaCl, 15 g KCl, 108 g Na_2HPO_4, 18 g KH_2PO_4, and 2.5 L H_2O.

From Autogen Bioclear:

Foetal Calf serum (FCS)	Batch 154-161200

From SIGMA Diagnostics:

Giemsa solution	Cat. No. 080K4365

From Santa Cruz Biotechnology:

anti-CDK4 (c-22) rabbit polyclonal lgG	Cat. No. L0407
anti-Cdc2 p34 (17) mouse monoclonal lgG2a	Cat. No. G0207

From Thermo Scientific:

Supersignal West pico chemiluminescent substrate Cat. No. 34080

From Promega:

CellTiter 96 ® Aqueous One Solution Reagent Cat. No. G3580

6.1.2) Cell lines and normal culture conditions:

a) Bladder transitional cell carcinoma cells (RT112 bladder cancer) (ECACC No. 850611) were cultured in nutrient mixture (Ham) F-12 supplemented with 10% v/v FCS, 1% v/v penicillin/streptomycin and 1% v/v glutamine at 37 °C in a humidified incubator containing 5% CO_2.

b) Immortalized normal fibroblasts (MRC5-hTERT fibroblast cells) were cultured in Dulbecco's modification of Eagle's (DMEM) supplemented with 10% v/v FCS, 1% v/v penicillin/streptomycin and 1% v/v glutamine at 37 °C in a humidified incubator containing 10% CO_2 [108].

6.1.3) Preparation of tissue culture mediums.

a) Nutrient mixture (Ham) F-12 medium was prepared from 500 mL of Ham's F-12 growth medium, 50 mL of FCS (10% of final volume), 5.0 mL of Penicillin/ Streptomycin (1% of final volume) and 5.0 mL of glutamine (1% of final volume). All of chemicals above were mixed together at room temperature. The medium was kept in the fridge and warmed up in water bath before use.

b) Dulbecco's Modification of Eagle's (DMEM) medium was prepared from 500 mL of DMEM growth medium, 50 mL of FCS (10% of final volume), 5.0 mL of Penicillin/ Streptomycin (1% of final volume) and 5.0 mL of glutamine (1% of final volume). All of chemicals above were mixed together at room temperature. The medium was kept in the fridge and warmed up in water bath before use.

6.2 GENERAL METHOD OF TISSUE CULTURE

6.2.1) Trypsinization (releasing cells from monolayer surface).

Cell lines that were maintained in tissue culture did not exceed 70% confluence. Hence exponentially growing supplies of cell lines were available for experiments. Trypsin-EDTA, FCS and growth medium were warmed to 37 °C before use and cells were typically replated in growth medium at 1:10 dilution.

The existing tissue culture medium was aspirated from cells that contained in tissue culture flask and washed with 10.0 mL of HBSS. Trypsin/EDTA solution (1.0 mL) was added to the tissue culture flask and incubated for 3-5 mins at 37 °C until all the cells had become detached. FCS 0.5 mL and tissue culture medium 10.0 mL were added to tissue culture flask, respectively. Cells suspension 9.0 mL was then transferred to another fresh sterile tube and left 1.0 mL of cells suspension which was typically replated in growth medium at 1:10 dilution.

6.2.2) Determination of total cell counts by using a hemocytometer.

For quantitative experiments accurate knowledge of the number of living cells in a culture is essential. This was performed as follows.

The 40.0 µL of cells suspension from section 6.2.1 was pipetted and transferred to hemocytometer. With the cover slip in place, cells were counted under microscope and average number of cells per square (n) was determined. The number of cells in 1 ml was then calculated by the use of equation: $n \times 10^4$. The appropriate dilutions were made to give different seeding concentrations.

6.3 CLONOGENIC ASSAY:

Clonogenic assay is a cell biology technique for studying the effectiveness of specific agents on the continued proliferation of cells. Under optimal growth conditions, a single cell suspension is seeded in tissue culture plastic. The cell is continuously divided and produced colonies. This assay is performed to obtain a correlation between the dose of peptide and number of cells dying [109].

6.3.1) Preparation of peptide solution.

The exactly amount of each peptide was weighed in eppendorf and dissolved in the tissue culture medium. The solution was sterilized by centrifuge at 11,000 rpm, 4 ^{o}C for 15 minutes. The peptide solution was transferred into another fresh eppendorf and left approximately 50 μL which might contain bacteria and fungi at the bottom of the first eppendorf. The peptide solution was then kept in ice all the time before use.

6.3.2) Plating of cells for clonogenic assay

The stock of cells suspension was counted (section 6.2.2), diluted in tissue culture medium and plated in 24-well dish (10-50 cells suspension per 250 μL of medium). Each peptide concentration was prepared (section 6.3.1) and added 250 μL in each well dish in quadruplicate. Plates were gently agitated to ensure an even cell distribution and incubated in a special incubator to obtain humidified atmosphere at 5 or 10% CO_2 depending on the cell lines. The special incubator (figure 1) is desired and used in this work which contained water at the bottom of the box and covered with parafilm. The tissue cultures plates are stood at the bottom of the box and kept in normal incubator. There is small space left at the top of our incubator which CO_2 can easily pass through our cells.

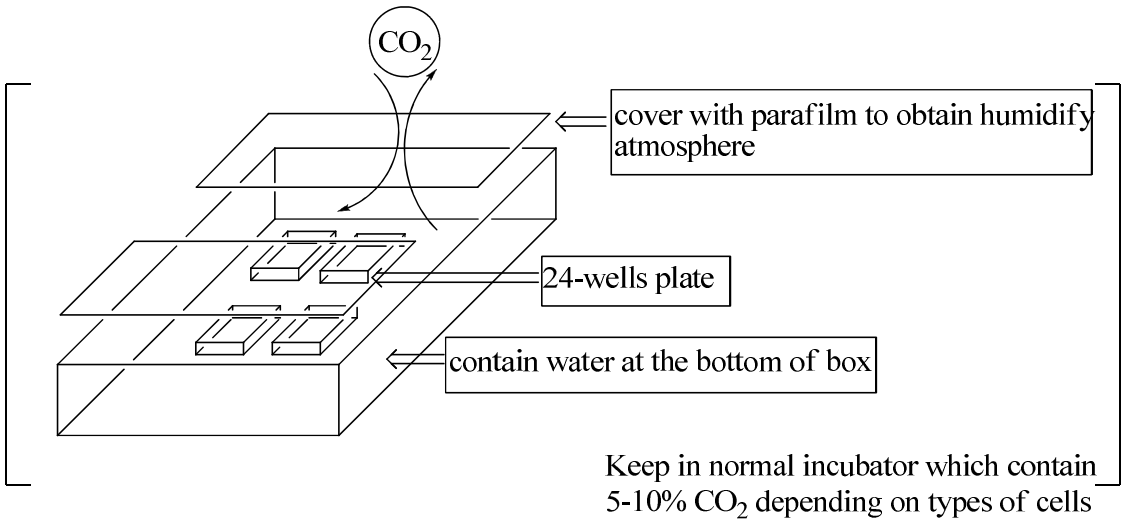


Figure 1: model of special incubator for biological assay.

6.3.3) Giemsa's staining protocol

After incubation, the tissue culture medium was aspirated from cultures and cells were carefully washed by 1.0 mL of cold PBS per one well. The PBS was aspirated and the cells

were fixed with 0.5 mL of MeOH for 5 minutes. All solvents were removed to waste bottle and then the plate was left to air dry. The cells were stained with 0.5 mL of giemsa solution (1: 20 dilution in deionised water) for 60 min on platform shaker. Solvent was removed to waste bottle and then left to air dry. The cells cultures could be visually examined under microscope for survival.

6.4 ASSAYS FOR 96 WELL PLATES

Cell proliferation and cell based ELISA assay are colorimetric method for determining the number of viable cells or the abundance of a particular antigen.

6.4.1) Cell proliferation assay

Cells suspension in tissue culture medium was seeded into 96-well culture plate and incubated at 37 °C in a special incubator which contained water itself to obtain humidified atmosphere at 5 or 10% CO_2 depending on the cell line. After incubation time, 100.0 µL of medium was refreshed and then 20 µL of CellTiter 96 ® Aqueous One Solution Reagent was added into each well dish. The 96-well plate was incubated at 37 °C in a humidified atmosphere of 5 or 10% CO_2 for 2 h. Absorbance was recorded at 490 nm by the Varioskan Flash instrument (using a 96-well plate reader) [110].

6.4.2) Cell based ELISA assay

Cells suspension in tissue culture medium was seeded into 96-well culture plate and incubated at 37 °C, 5 or 10% CO_2 in a special incubator. After incubation time, medium was aspirated from the cells and 100.0 µL of PBS was added to wash medium off. 4% *p*-Formaldehyde/PBS was freshly prepared and added (100 µL) in each well to fix the cells. The plate was incubated at room temperature for 15 min and washed with PBS/0.1% Triton X-100 to permeabilize the cells three times. 10% FCS/PBS 100 µL was added in each well and incubated for 1 h at room temperature to block non-specific sites. Anti-CDK1 (primary antibody) was diluted with 10% FCS/PBS (blocking buffer) and added (25.0 µL) in each well. The plate was incubated at room temperature for 1 h. The solution was removed to waste bottle and washed with 100.0 µL of PBS/0.1% Triton X-100 three times to permeabilize the cells. Horse radish peroxidase conjugated (secondary antibody) was prepared in 10% FCS/PBS (blocking buffer) and added (50.0 µL) in each well. The plate

was incubated at room temperature for 1 h and washed with 100.0 μL of 10% FCS/PBS (blocking buffer) two times. Then, cells were washed with 100.0 μL of PBS three times. The chemoluminescent substrate solution was prepared by the mixture between equal amount of luminol enhancer and stable peroxide reagent. The solution was protected from light, left at room temperature and then added 100.0 μL in each well. The luminescence was recorded by the Varioskan Flash instrument (using a 96-well plate reader).

CHAPTER 7: BIOLOGICAL ASSAYS DEVELOPMENT

7.1) CELL BASED ELISA ASSAY DEVELOPMENT

The enzyme-linked immunosorbent assay (ELISA) is one of biochemical technique that used mainly in immunology to detect the presence of an antibody or an antigen in a sample especially for drug development [111, 112].

From the previous study, Warenius, H. found that THR1 could make an increasing of CDK1 which was a cancer marker [5]. Therefore, we used this knowledge for a cell based ELISA assay. Our observations suggested that the cell based ELISA assay could be a useful method for drug development and chemosensitivity assessment because this assay required small amount of peptides and used short incubation time. Thus, the objectives of this work were to develop the cell based ELISA assay and to investigate anticancer activity of THR1 [113]. In this work, we used anti-CDK1 as a primary antibody and horse radish peroxidase conjugated as a secondary antibody to determine immunoreactivity of cell growth in 96-wells plate. Between each step the plate was typically washed with a mild detergent solution to remove any proteins or antibodies that were not specifically bound. After the final wash step the plate was developed by adding a chemoluminescent (luminal-peroxide substrate) substrate solution to produce a visible signal, which indicated the quantity of antigen in the sample. These assays employed luminescence substrate. An enhanced luminal-peroxide substrate solution was added to each well to produce a chemical luminescence light [114]. A briefly mechanism is shown in figure 2.

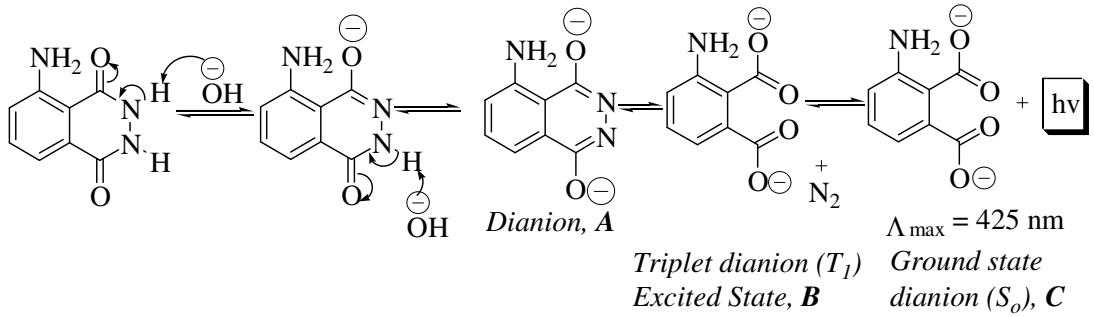


Figure 2: Oxidation of luminol by stable peroxide reagent.

The luminol is converted by the basic solution into the resonance-stabilized dianion **A**, which is oxidized by stable peroxide reagent into dicarboxylate ion **B**, accompanied by the

loss of molecular nitrogen (N_2) gas. The molecule **B** is formed in an excited state (higher energy), and its extra energy is emitted from the molecule in a form of luminescence light and allowed the molecule to go to its ground state form **C**. Amount of luminescence was easily measured in the λ_{Max} by the Varioskan Flash instrument (using a 96-well plate reader).

CELL BASED ELISA ASSAY 1: an initially assay development.

The objectives of this assay were to develop and optimize suitable conditions for cell based ELISA assay (control experiment and without any peptide).

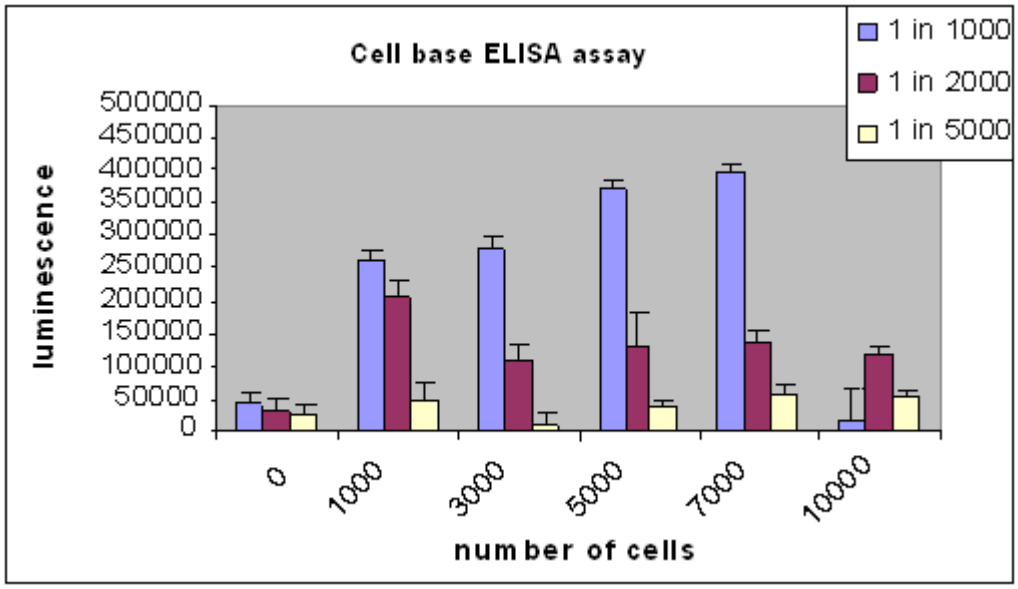


Figure 3: *The graph was plotted between number of bladder cells and chemical luminescent that was recorded from standard plate reader at 0.1 second per well. RT112 bladder cancer cells were trypsinized and seeded at 1000, 3000, 5000, 7000 and 10000 cells suspension in Ham's F-12 medium 100 µL. Plate were kept in incubator at 37 °C, 5% CO_2 for 24 h. Anti-CDK1 (diluted in 10% FCS/PBS) and horse radish peroxidise (diluted in 10% FCS in PBS) were used as primary and secondary antibody, respectively. The control was done without any cells. Concentration of secondary antibody was varied from 1:1000, 1:2000 and 1:5000 in 10% FCS/PBS. The chemoluminescent substrate solution was added into each well and 96-well plate was scanned by luminescence (relative light units, RLU) on standard plate reader at 0.1 second per well.*

As result from graph, we found that amount of chemical luminescent was not related with number of cells.

CELL BASED ELISA ASSAY 2: repeat the initial assay development.

The aim of this assay was to repeat the initial assay under the same conditions as described in previous assay.

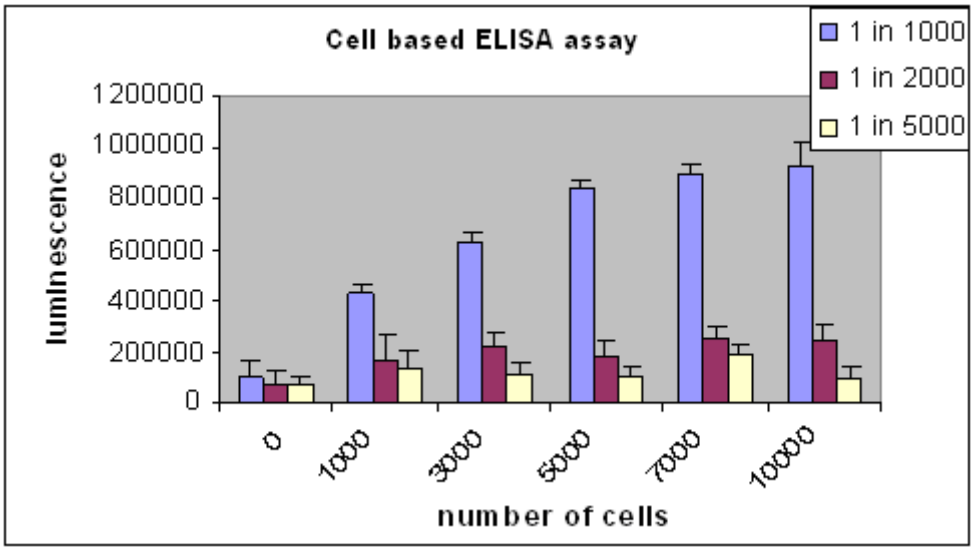


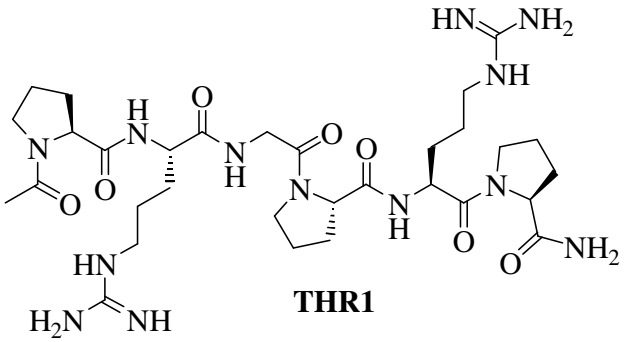
Figure 4: *The graph was plotted between number of bladder cells and chemical luminescent that was recorded from standard plate reader at 0.1 second per well. RT112 bladder cancer cells were trypsinized and seeded at 1000, 3000, 5000, 7000 and 10000 cells suspension in Ham's F-12 medium 100 µL. Plate were kept in incubator at 37 °C, 5% CO_2 for 24 h. Anti-CDK1 (diluted in 10% FCS/PBS) and horse radish peroxidise (diluted in 10% FCS in PBS) were used as primary and secondary antibody, respectively. The control experiment was done without any cells. Concentration of secondary antibody was varied from 1:1000, 1:2000 and 1:5000 in 10% FCS/PBS. The chemoluminescent substrate solution was added into each well and 96-well plate was scanned by luminescence (relative light units, RLU) on standard plate reader at 0.1 second per well.*

As result, we found that amount of chemical luminescent was related with number of cells and the suitable dilution of secondary antibody (horseradish peroxide) was 1:1000 of 10% FCS in PBS.

CELL BASED ELISA ASSAY 3: THR1 testing against RT112 bladder cancer cells.

The objective of this assay was to investigate anticancer activity of THR1 against RT112 bladder cancer cells by cell based ELISA assay under the same conditions that was optimized from the previous assays.

Details: Cells were trypsinized and plated at 50 cells suspension per 100 µL of Ham's F-12 medium into 96-well tissue culture plates. The number of cells was decreased from previous assay to 50 cells in each well to prevent confluence of cells on day 10. THR1 is a hexapeptide that is supported from Theryte Ltd and chemical structure is shown in figure 6. Stock solution of THR1 (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) was prepared in Ham's F-12 medium and added (100 µL) into each well (6.3.1). The final concentration of THR1 was 3 mM (0.43 mg/200 µL) and 5 mM (0.72 mg/200 µL) per 200 µL of medium. The cells were allowed to grow in medium for control experiment (without any peptide). This assay was performed in triplicate. The first two plates were measured by cell based ELISA assay which was described in section 6.4.2 on day 3, 6, 8 and 10. Chemoluminescent substrate solution was prepared by mixing equal amount of luminol enhancer and stable peroxide reagent and added 100.0 µL into each well. The luminescence was recorded by the Varioskan Flash instrument (using a 96-well plate reader). The third plate was measured by cell proliferation assay (6.4.1) on day 3, 6, 8 and 10 by using CellTiter 96 Aqueous OneSolution cell proliferation assay reagent (Promega) and recorded absorbance at 490 nm by the Varioskan Flash instrument (using a 96-well plate reader).



THR1

Figure 5: Chemical structure of THR1.

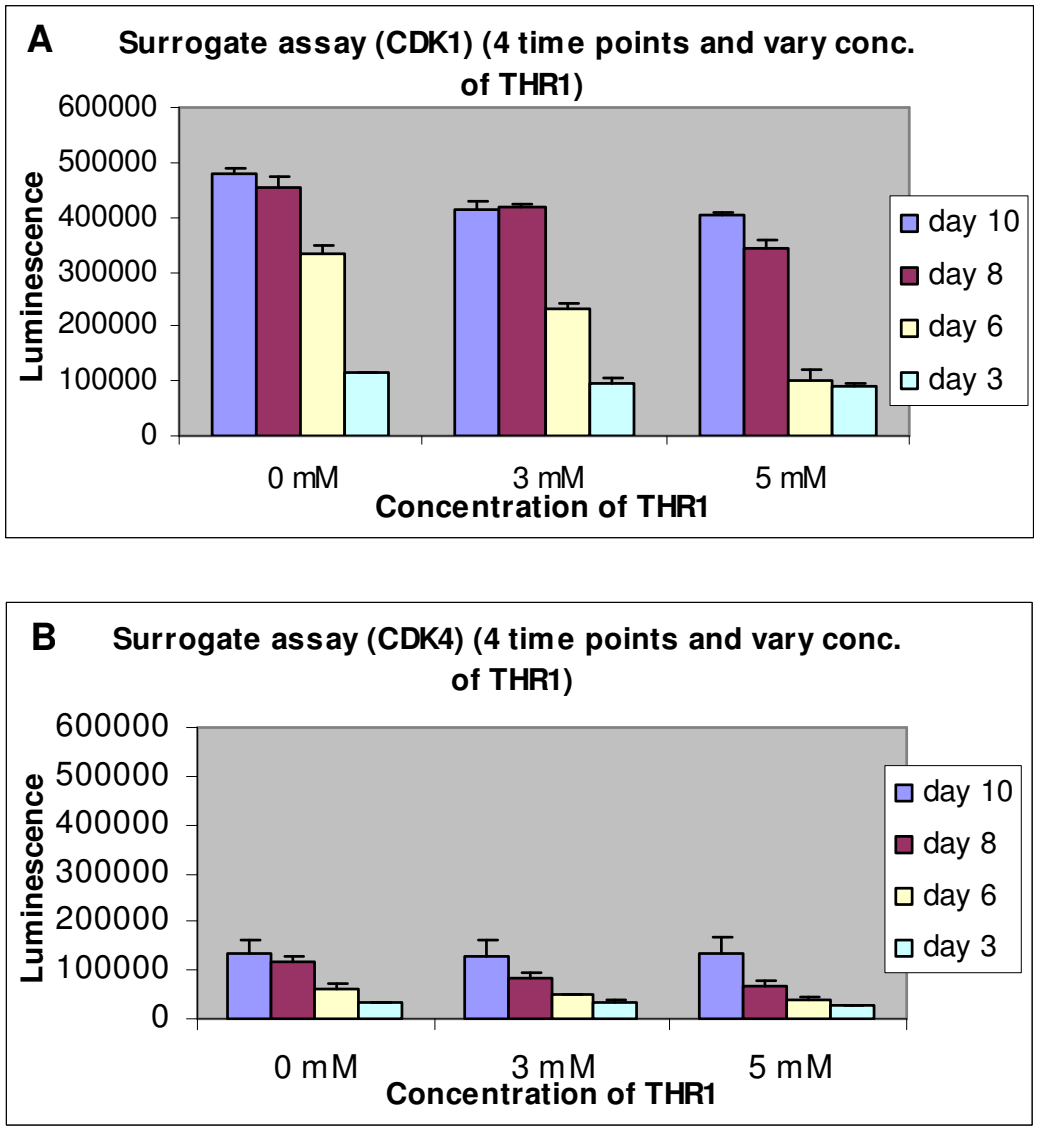


Figure 6: *RT112 bladder cancer cells were trypsinized and seeded at 50 cells suspension in Ham's F-12 medium 200 µL (total volume). Plate were allowed to expose with THR1 at 0 (for control assay), 3 and 5 mM and kept in incubator at 37 °C, 5% CO_2 for 3, 6, 8 and 10 days. Either CDK1 or CDK4 that was used as primary antibody was diluted in 10% of FCS/PBS and added 100 µL into each well. Horse radish peroxidase was diluted in 10% FCS in PBS and used as secondary antibody. The chemoluminescent substrate solution was added into each well and the plate was scanned by luminescence (relative light units, RLU) on standard plate reader at 0.1 second per well. The graph was plotted between concentration of THR1 and chemical luminescent.*

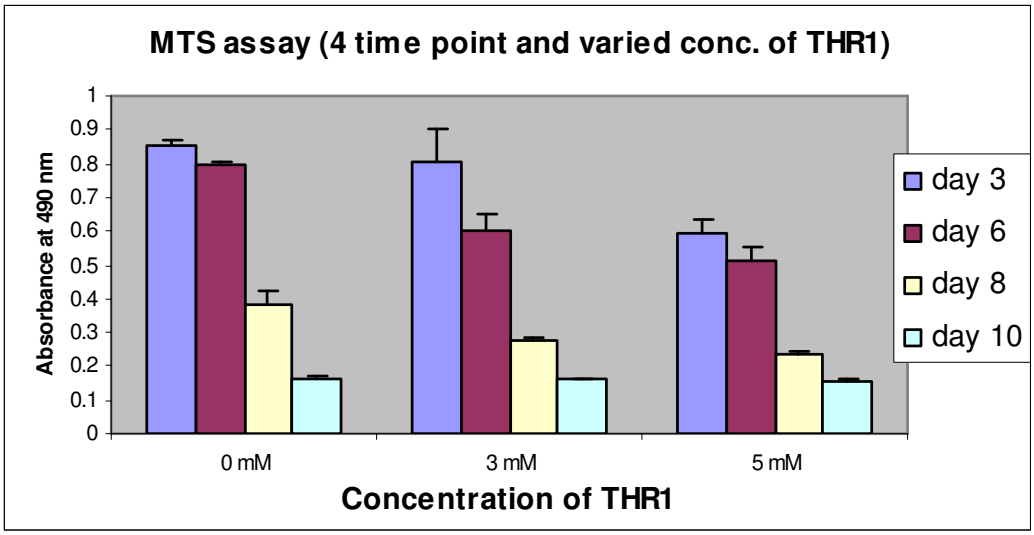


Figure 7: *RT112 bladder cancer cells were trypsinized and seeded 50 cells in 100 µL of Ham's F-12 medium. Plate were exposed to THR1 at 0 (for control assay), 3 and 5 mM and kept in incubator at 37 °C, 5% CO_2 for 3, 6, 8 and 10 days. Cell proliferation was assayed in MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium or Owen's reagent).*

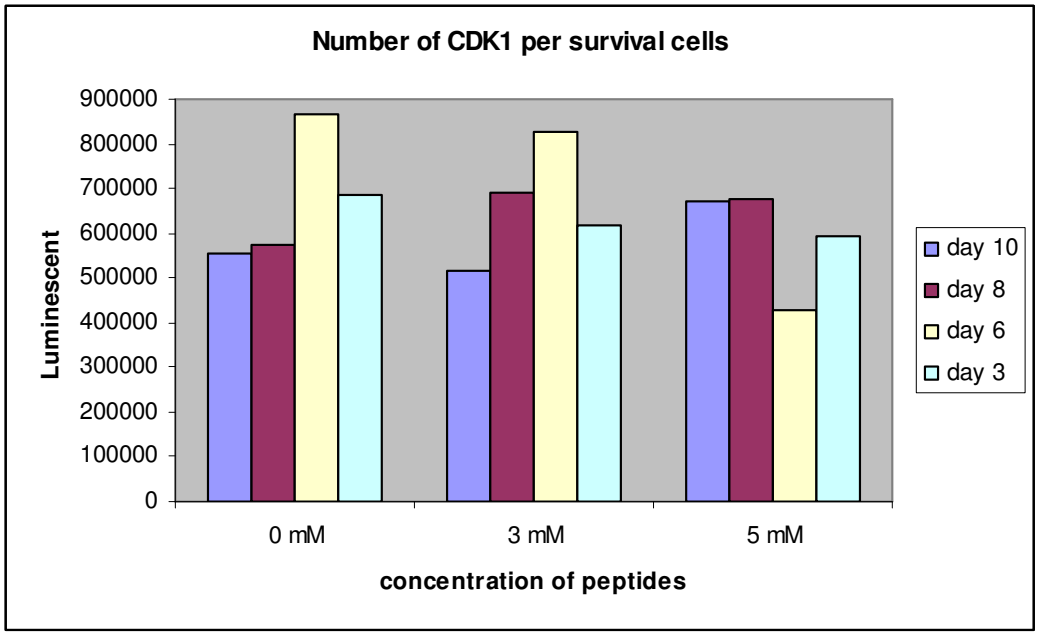


Figure 8: *Number of CDK1 per survival cells was measured by plot graph between luminescent which divided by number of cells from MTS assay and concentration of peptide THR1 at 0 (for control assay), 3 and 5 mM.*

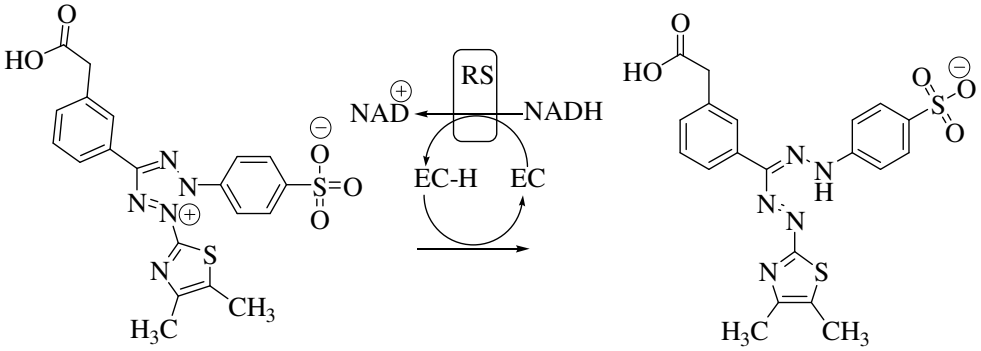
In conclusion, cell based ELISA assay did not show any significant results between THR1 and either CDK1 or CDK4. When THR1 increased, CDK1 or CDK4 did not increase. So, both of these CDK1 and CDK4 could not be used as surrogate materials for this assay [*].

** In comparison with the parallel study from our laboratory with cyclic peptide (THR53) under the same conditions, the results did not show any relationship between CDK1 and the peptide THR53 as well. Therefore this assay failed to determine anticancer activity of THR1 and surrogate assay was abandoned.*

7.2) CELL PROLIFERATION ASSAY DEVELOPMENT

Cell proliferation assay performs by adding a small amount of the CellTiter 96® Aqueous One Solution Reagent directly to culture wells, incubating for 1-4 hours and then recording the absorbance at 490 nm with a 96-well plate reader [115].

To investigate the suitable biological assay for our synthetic peptide, the cell proliferation assay was studied. A colorimetric method uses to determine the number of viable cells in proliferation assay. The assay is straight-forward, simple method and small amount of peptide is required. Generally, a tetrazolium compound **A** can be reduced to a colored formazan product that is directly relative to the number of living cells. Therefore, the quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. The reaction of formazan formation is shown in figure 9.



Tetrazoliaum compound **A** Formazan

Figure 9: The reaction of formazan formation.

In principle, tetrazolium salt **A** is cleaved by the succinate-tetrazolium reductase (EC) to form soluble formazan dye, which exists in mitochondrial respiratory chain and is activated

only in viable cells. The total activity of this mitochondrial dehydrogenase in a sample rises with the increase of number of viable cells. As the increase of enzyme activity leads to an increase of the production of formazan dye, then the quantity of formazan dye is related directly with the number of metabolically active cells in the medium. The formazan dye formed by metabolically active cell can be quantitated by measuring its absorbance. The absorbance of formazan dye solution is in direct proportion to the number of viable cells [115]. This product is designed for spectrophotometric quantification of cell growth and proliferation assay.

CELL PROLIFERATION ASSAY 1: an initially assay development.

The objectives of this assay were to develop suitable conditions for cell proliferation assay and also to investigate anticancer activity of THR1 (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) against RT112 bladder cells.

Details: The cells were trypsinized and plated 50, 100 and 200 cells in 96-well plates. The procedure was performed in tripicate. Stock solution of THR1 (10.0 mM) was prepared in Ham's F-12 medium at 0 °C and added into each well dish. The cells were exposed to THR1 for 25 days at 37 ^{o}C in 5% CO_2 in the incubator. The final concentration of THR1 was 5.0 mM (0.72 mg/200 μL) in 200 μL of medium. Cell proliferation was assayed on day 15, 20 and 25 by adding 200 μL of the CellTiter 96® Aqueous One Solution Reagent (Promega) directly to culture well, incubated and then recorded absorbance at 490 nm by the Varioskan Flash instrument (using a 96-well plate reader). Cell proliferation was performed as describe in section 6.4.1 on day 15, 20 and 25.

Result on day 15 is shown in figure 10. The CellTiter 96® Aqueous One Solution Reagent 20 μL was added in each well and allowed to react with bladder cell. The incubation time was varied from 1, 2 and 3 h. Absorbance of these solutions was monitored at 490 nm. We found that for 1 h of incubation time the CellTiter 96® Aqueous One Solution Reagent did not completely react with RT112 bladder cells then the recorded absorbance at 490 nm was lower than 2 and 3 h of incubation time. We also found that the incubation time of tissue culture plate for 2 h showed the same significant results as 3 h then the suitable incubation time for our work was fixed for 2 h.

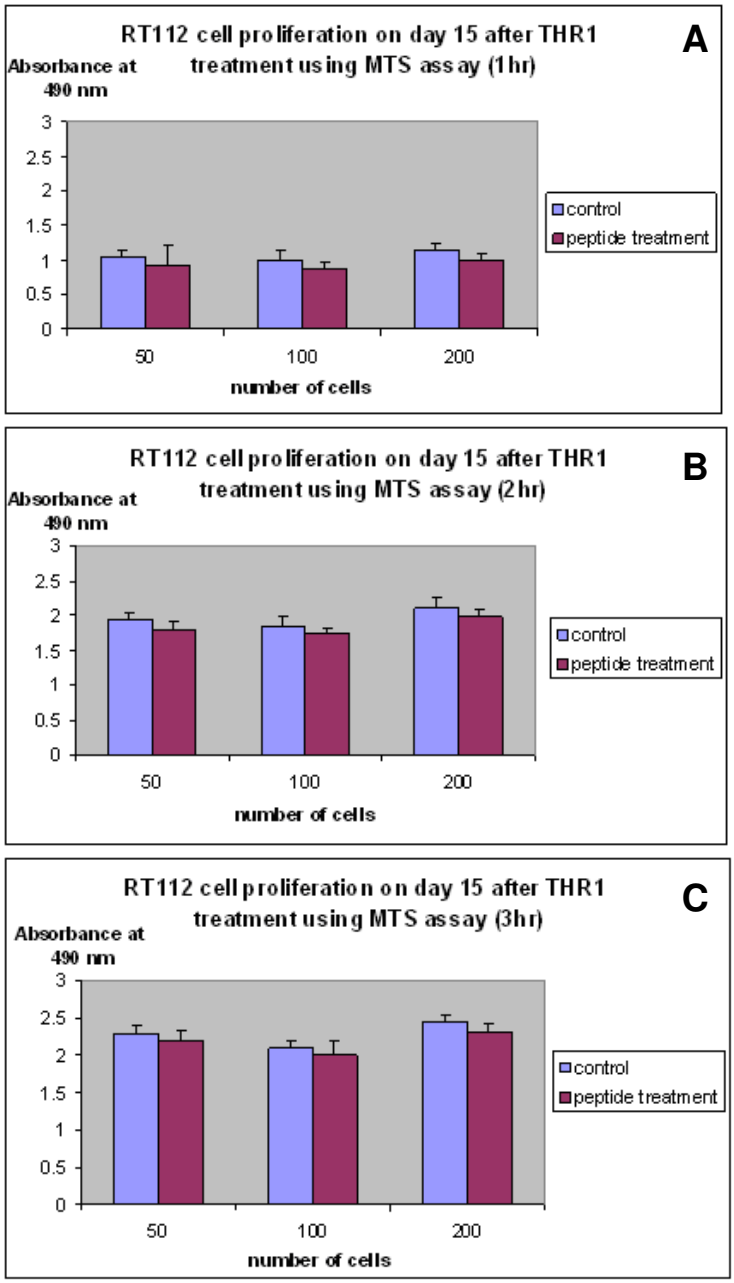


Figure 10: *RT112 bladder cancer cells were treated with and without THR1 (5.0 mM per 200 µL). Number of cells was varied from 50, 100 and 200 cells in Ham's F-12 medium. THR1 (5 mM = 0.72 mg/200 µL) was prepared and treated with RT112 cells. The cells were grown with and without THR1 solution for 15 days. Cell proliferation assay was performed by the CellTiter 96® Aqueous One Solution Reagent and varied incubating time from 1, 2 and 3 at 37 °C, 5% CO_2. The graph was plotted between number of bladder cells and absorbance at 490 nm.*

The results of cell proliferation assay on day 15, 20 and 25 are shown in figure 11. On day 15, the number of cells with THR1 treatment was slightly decreased when we compared with the controlled experiment. There were some significant of results at 100 cells on day 20 and 200 cells on day 25. THR1 was shown better anticancer activity when we treated with 50 and 100 cells. However, there were too many cells and some cells started to die on day 25 especially when we plated 200 cells into each well dish.

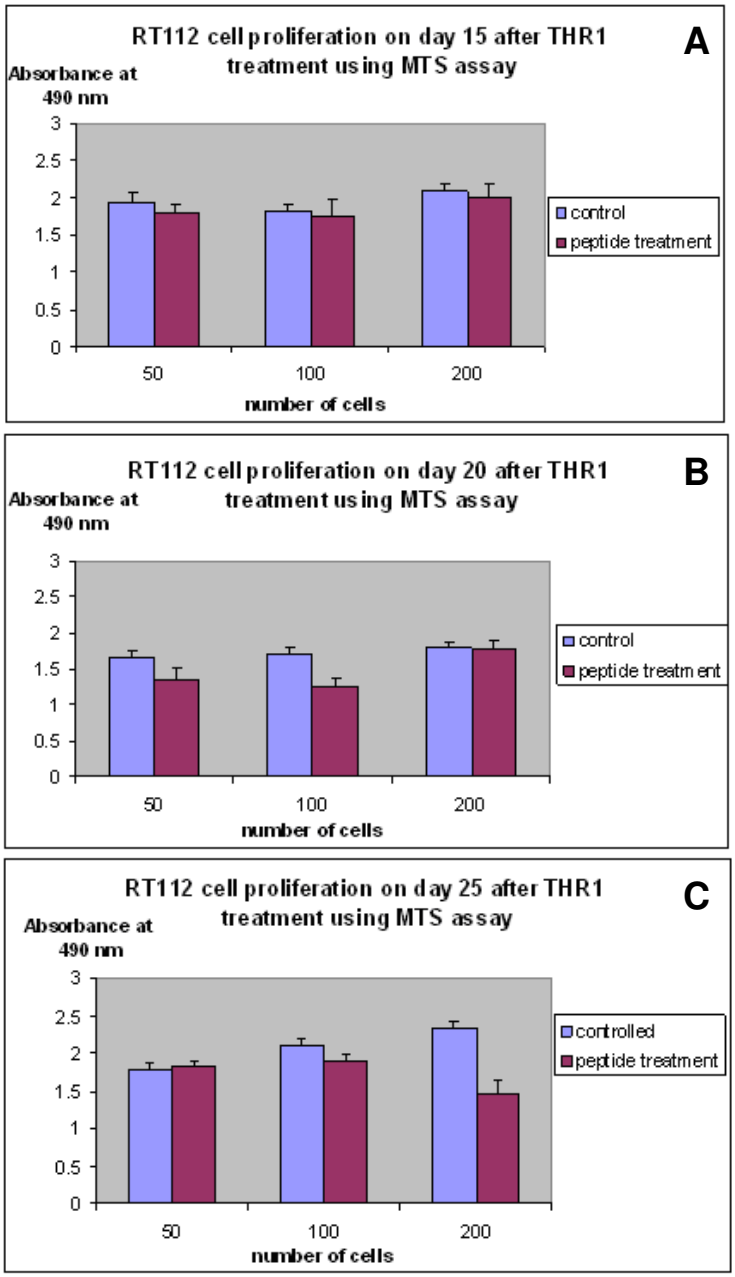


Figure 11: *RT112 bladder cancer cells were treated with and without THR1 (5.0 mM per 200 μL). Number of cells was varied from 50, 100 and 200 cells in Ham's F-12 medium. THR1 (5 mM = 0.72 mg/200 μL) was prepared and treated with RT112 cells. The cells were grown with and without THR1 solution for 15, 20 and 25 days. Cell proliferation assay was performed by the CellTiter 96® Aqueous One Solution Reagent and varied incubating time from 1, 2 and 3 at 37 °C, 5% CO_2. The graph was plotted between number of bladder cells and absorbance at 490 nm.*

CELL PROLIFERATION ASSAY 2: determine of number of cells by calibration curve.

According to the previous cell proliferation assay, there are too many cells on day 25. Then the objective of this assay was to find suitable number of cells density in each well by calculating from calibration curve which was plotted between absorbance at 490 nm and number of cells.

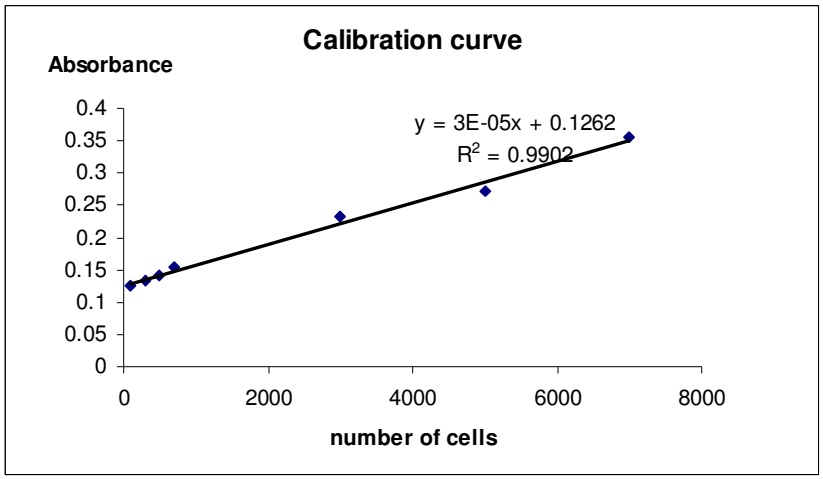


Figure 12: *Cells were trypsinized and seeded at 100, 300, 500, 700, 1000, 3000, 5000, 7000, 10000 and 20000 cells in 200 µL of Ham's F-12 medium in each well of 96-well plates and allowed to incubate at 37 ^{o}C in 5% CO_2 for 24 h. Cell proliferation performed by CellTiter 96 Aqueous One Solution cell proliferation assay reagent (Promega) and incubated for 2 h in incubator at 37 °C, 5% CO_2 before recorded by the Varioskan Flash instrument (using a 96-well plate reader). Calibration curve of number of RT112 bladder cancer cells was plotted between number of cells and absorbance at λ 490 nm.*

As a result, maximum absorbance at 0.35 correlated with 7,000 cells and did not cover number of cells from the first cell proliferation assay. Then we could not calculated total number of cell on day 15.

CELL PROLIFERATION ASSAY 3: extend the calibration curve by increasing number of cells.

The objective of this assay was to determine number of cells density in each well dish by the calculation from calibration curve of absorbance. The method and conditions in this assay were repeated from cell proliferation assay 2.

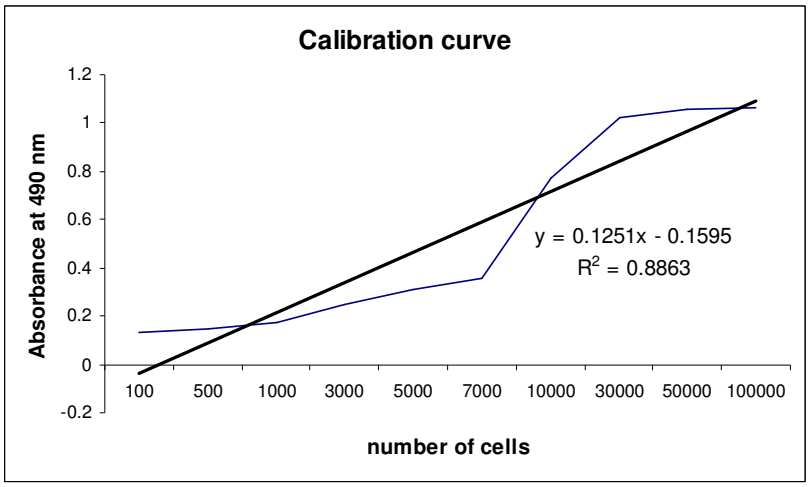


Figure 13: *Calibration curve showed number of RT112 bladder cancer cells, related with absorbance at λ 490 nm. Cells were trypsinized and seeded at 100, 500, 1000, 3000, 5000, 7000, 10000, 50000, 70000 and 100000 cells/200 µL of Ham's F-12 medium in each well dish in 96-well plates and allowed to incubate at 37 ^{o}C in 5% CO_2 for 24 h. Cell proliferation was performed by CellTiter 96 Aqueous One Solution cell proliferation assay reagent (Promega) and incubated for 2 h in incubator at 37 °C, 5% CO_2 before recorded by the Varioskan Flash instrument (using a 96-well plate reader). Calibration curve of number of RT112 bladder cancer cells was plotted between number of cells and absorbance at λ 490 nm.*

As a result, the calibration curve was plotted between number of cells and absorbance at 490 nm (figure 13). The absorbance that continuously increased from 0.1 to 0.4 related with number of cells from 100 to 7,000 cells (same result with cell proliferation assay 2). When we increased number of cells from 7000 to 40000 cells, the absorbance dramatically increased from 0.4 to 1.1. The maximum absorbance at 1.1 related to 100,000 cells per well. In comparison, the cells number in this calibration curve did not cover the maximum amount of cells in the cell proliferation assay 1.

In conclusion, according to cell proliferation assay 1, 2 and 3, we found that if we plated 50, 100 and 200 cells suspension in 100 µL into each well, the number of cells increased more than 100,000 cells on day 15. Then, THR1 did not show anticancer activity because there were too many cells density in each well dish.

CELL PROLIFERATION ASSAY 4: decrease number of cells density in each well dish.

The objective of this assay was to optimize cells density in each well dish by decreasing number of cells to 10, 30 and 50 cells under the same conditions which was developed from previous assay.

Details: The cells were trypsinized and plated at 10, 30 and 50 cells suspension per 200 µL of Ham's F-12 medium into 96-well tissue culture plates. The cells were allowed to grow for maximum 25 days at 37 ^{o}C in 5% CO_2 in the incubator. Cell proliferation assay was performed on day 15, 20 and 25. The absorbance of cell proliferation on day 15, 20 and 25 was measured at λ 490 nm. The graphs were plotted and results compared between control experiment 1 and 2 under the same conditions (figure 14). When the number of cells was increased, measured absorbance was increased in the same trend. Especially on day 20 and 25 (30 and 50 cells), cells were overcrowded and column graph was constant.

As result from Warenius, H. found that the suitable incubation time for this cell proliferation assay was 25 days. Our observation, we found that cells were fulled on day 20-25, although we started with as little as 10 cells in each well. Then, from cell proliferation assay development, we concluded that this assay was not suitable for assaying our drugs.

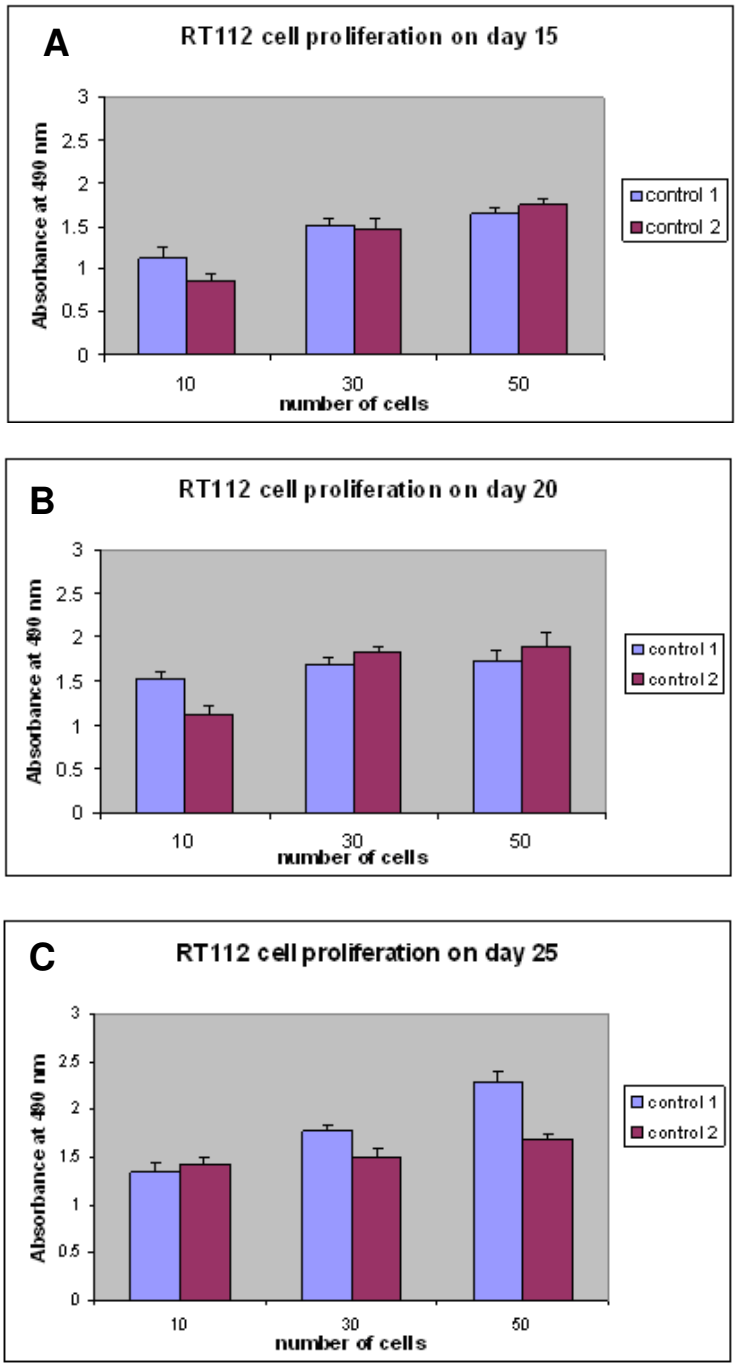


Figure 14: *The graph was plotted between number of RT112 cells and absorbance at 490 nm. The cells were trypsinized and plated at 10, 30 and 50 cells in 96-wells plate. The cells were grown for maximum 25 days at 37 ^{o}C in 5% CO_2 in the incubator. This assay was performed in duplicate at the same plate (for control assay 1 and 2) and each plate was repeated in triplicate under the same conditions. Cell proliferation was performed on day 15, 20 and 25.*

7.3) CLONOGENIC ASSAY DEVELOPMENT

Clonogenic assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony.

CLONOGENIC ASSAY 1: an initially assay development.

The objectives of this assay were to optimize conditions for clonogenic assay and study anticancer activity of THR1 against RT112 bladder cancer cells.

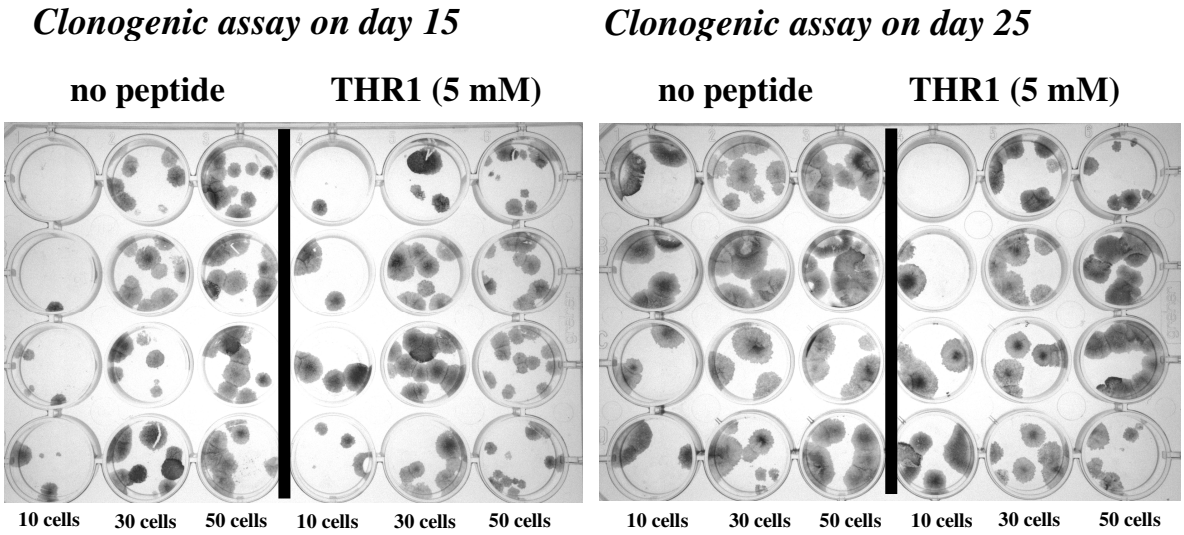
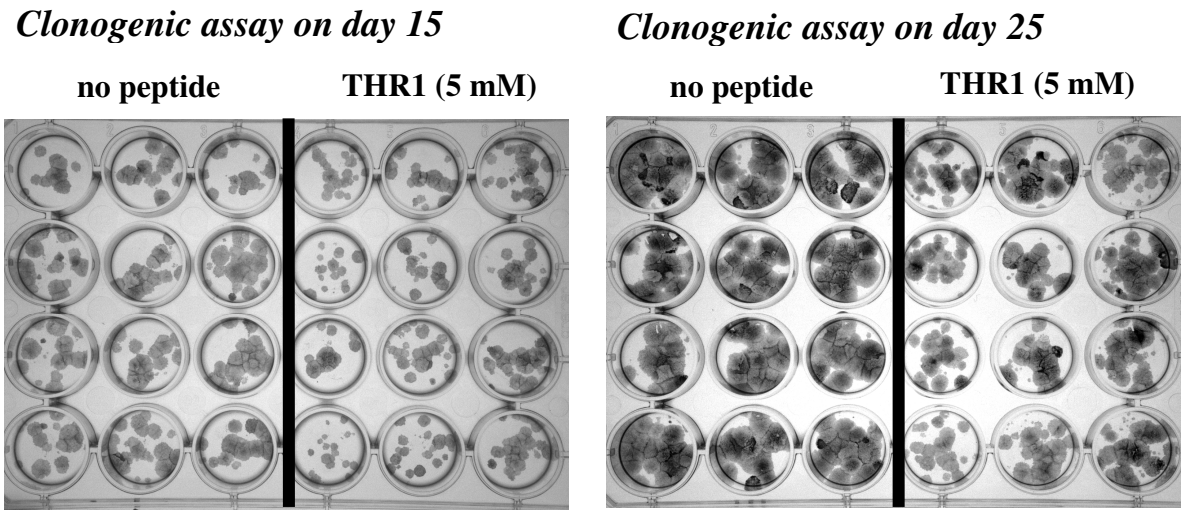


Figure 15: *Pictures of colony forming of RT112 bladder cancer cells on day 15 and 25. The bladder cells were trypsinized and seeded at 10, 30 and 50 cells in 24-well plate. The RT112 cells were allowed to grow in 500 µL of THR1 (5 mM = 1.8 mg/500 µL). The results were compared with controlled experiment (without peptide). This assay was performed in duplicate under the same conditions and kept at 37 ^{o}C in 5% CO_2 in the incubator. After 15 and 25 days, the cells were fixed with MeOH, stained with Giemsa's solution and visually examined under microscope for survival. The Giemsa's staining protocol is described in section 6.3.3.*

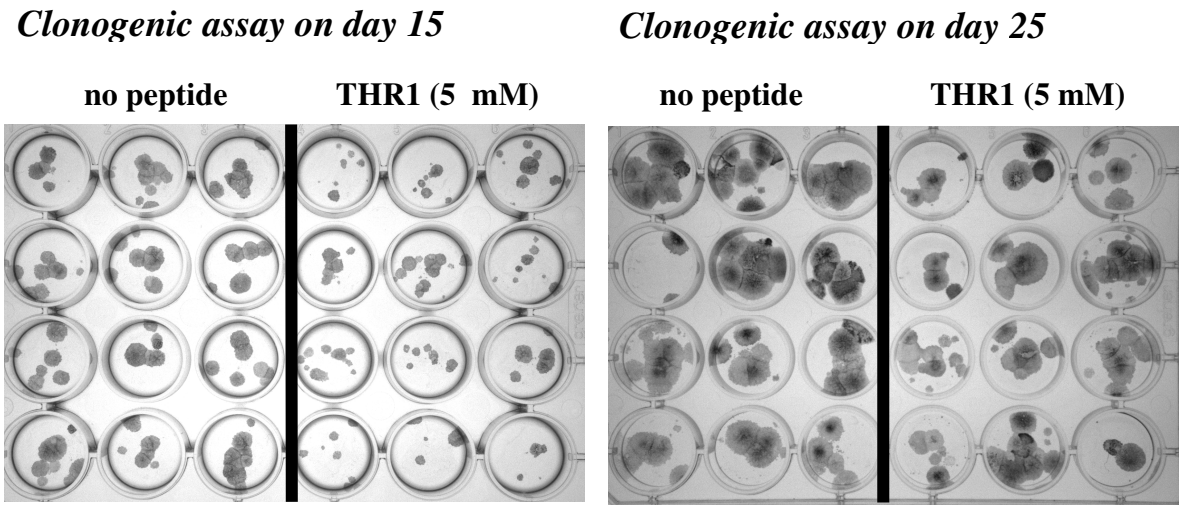
As results (figure 15), if we plated 50 cells per well, there were too many cells after day 15. Number and size of RT112 colony showed the same results in with and without THR1. Therefore suitable number of cells density per well dish was either 10 or 30 cells per 500 µL of tissue culture medium or peptide solution.

CLONOGENIC ASSAY 2: repeat an initially assay.

The objectives of this assay were to optimize and find the suitable of cell density (between 10 or 30 cells) in each well dish.



A*: 30 cells suspension in each well dish.*



B: *10 cells suspension in each well dish.*

Figure 16: *Pictures of colony forming of RT112 bladder cancer cells on day 15 and 25. The cells were trypsinized and seeded at 30 cells (A) and 10 cells (B) in 24-well plate. The RT112 cells were allowed to grow in 500 µL of THR1 (5 mM = 1.8 mg/500 µL). The results were compared with control experiment (without peptide). This assay was performed in duplicate under the same conditions and kept at 37 ^{o}C in 5% CO_2 in the special incubator. After 15 and 25 days, the cells were fixed with MeOH, stained with Giemsa's solution and visually examined under microscope for survival.*

Figure 16A: cells were trypsinized and plated at 30 cells per well. The results were compared between with and without THR1 treatment and we found that number and size of RT112 colonies were similar on day 15. Result on day 25, there were too many cells in control wells (without THR1) and also size of colonies was bigger than size of colonies with THR1 treatment. In parallel, results are shown in figure 16B: cells were trypsinized and plated at 10 cells per well. We found that the results on day 15 were similar in both of control and peptide treatment. Result on day 25, we found that THR1 had no effect with RT112 bladder cancer cells. Because of size of colonies in THR1 treatment were smaller than size of colonies without peptide.

In conclusion, from clonogenic assay 1 and 2, we found that the suitable cell density per well was 10 cells/500 µL of either THR1 solution or medium (control experiment) in each well dish. The quantity of cells per well and amount of peptide solution that used in this assay (10 cells/500 µL) was sensitive for long term assay (maximum 25 days). Then to solve this problem, special incubator was desired to use in this assay. The incubator was used to protect the evaporation of medium or peptide solution from cells (described in section 6.3.2). We also found that THR1 was shown slightly anticancer activity on day 25. So, in the next assay, RT112 cells were allowed to expose with THR1 longer (maximum 30 days) until this peptide showed the better activity.

CLONOGENIC ASSAY 3: THR1 and THR2 testing against RT112 bladder cells (longer incubation time).

The objectives of this assay were to optimize conditions and determine suitable concentration of THR1 and THR2 by vary concentration from 2, 3, 4 and 5 mM. The comparison of chemical structure between THR1 and THR2 are shown in figure 17. The anticancer activity of THR1 was studied and compared the results with THR2 which was used as a negative control peptide for this clonogenic assay.

Details: Peptide drugs, THR1 (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) and THR2 (*N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro-NH_2) were prepared in Ham's F-12 medium at 0 °C in different concentration from 2 (0.7 mg/500 µL), 3 (1.1 mg/500 µL), 4 (1.4 mg/500 µL), and 5 (1.8 mg/500 µL) mM.

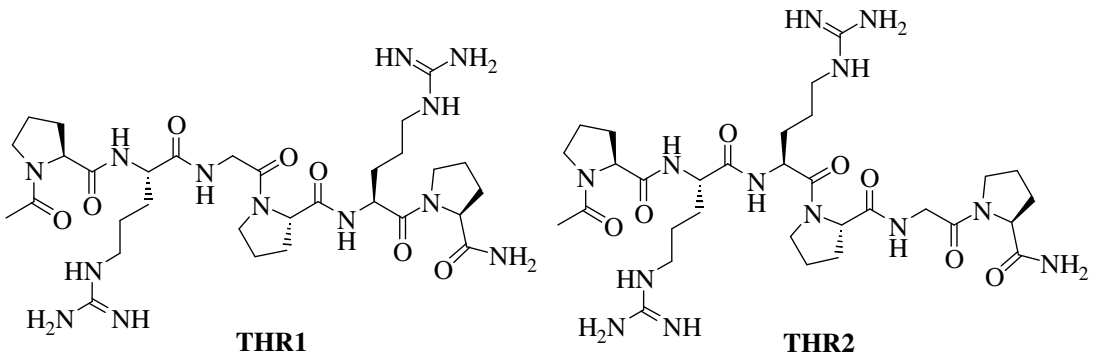


Figure 17: Chemical structure of THR1 (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) and THR2 (*N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro-NH_2).

As results from previous assay, THR1 had no anticancer activity against bladder cells on day 25. This assay, the incubation time was optimized by leaving tissue culture plates in the special incubator longer (maximum 30 days). The colony forming of bladder cells was observed daily after day 25 until death was seen. We found that THR1 killed all cells on day 29. The results of colony forming are shown in figure 18. The RT112 cells that were treated with 5 mM of THR1, started to die on day 25 and completely dead on day 29. All of tissue culture plates were kept in special incubator to prevent evaporation of medium or peptide solution (described in section 6.3.2). When the concentration of THR1 decreased, ability of cells survival increased. A comparison result between THR1 and THR2 (negative control peptide), we found that THR2 did not show any effect with bladder cells at all concentrations.

In conclusion, the results of our study indicated that 10 cells per 500 μL of medium (for control experiment) or peptide solution (5 mM) were suitable cells density for our clonogenic assay. The tissue culture plates were kept at 37 ^{o}C in 5% CO_2 in special incubator for 29 days.

Clonogenic assay

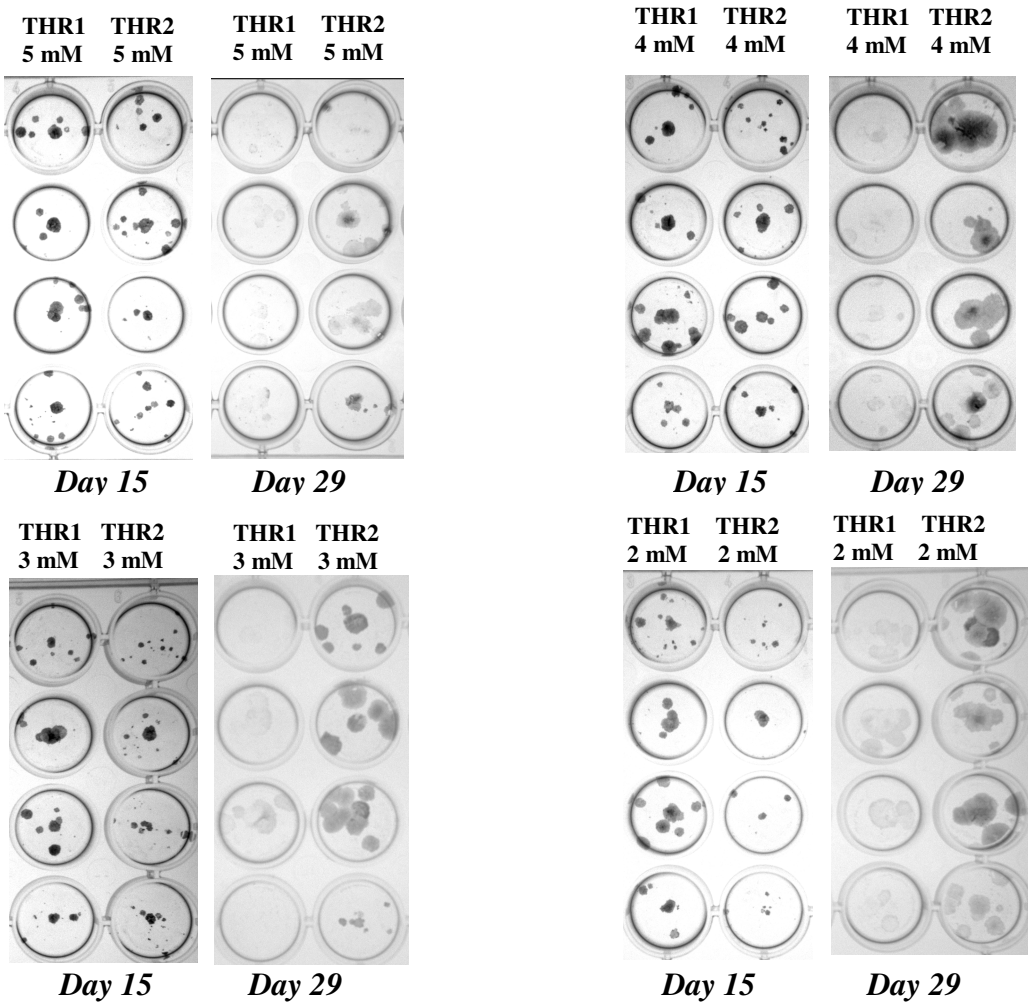


Figure 18: *Pictures of colony forming of RT112 bladder cancer cells on day 15 and 29. The bladder cells were trypsinized and seeded at 10 cells in each well dish of 24-well plate. The anticancer activity was studied when cells exposed with THR1 and THR2. The concentration of both peptides was varied from 5, 4, 3, 2 mM (500 µL of total volume). This assay was performed in duplicate under the same conditions and kept at 37 ^{o}C in 5% CO_2 in the special incubator. After 15 and 29 days, the cells were fixed with MeOH, stained with Giemsa's solution and visually examined under microscope for survival.*

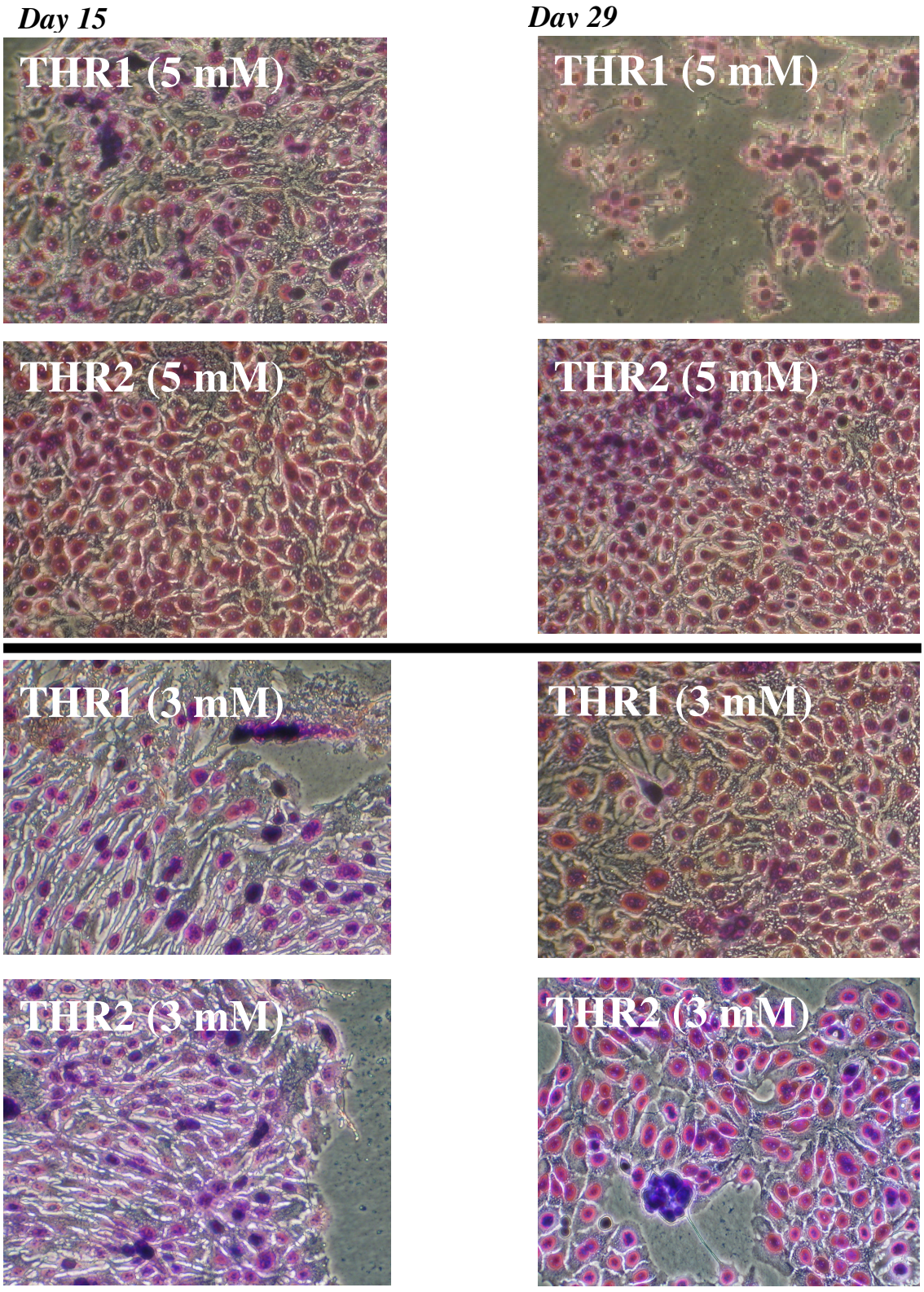


Figure 19: *Morphological appearances of RT112 cells following exposure to THR1 and THR2 with indicated concentration on day 15 and 29.*

CLONOGENIC ASSAY 4: repeat THR1 and THR2 testing against RT112 bladder cells.

The objectives of this assay were to repeat THR1 and THR2 treatment under the same conditions using in clonogenic assay 3. The concentration of both peptides were varied from 5, 4, 3, 2 mM and treated against RT112 cells.

Details: Anticancer activity of THR1 was studied by clonogenic assay against RT112 cells and results were compared with THR2 treatment that was used as a negative control peptide. The cells were allowed to grow in tissue culture medium 500 μL for control experiment. THR1 solutions were prepared by diluted with Ham's F-12 medium and concentration of peptide was varied from 2 (0.7 mg/500 μL), 3 (1.1 mg/500 μL), 4 (1.4 mg/500 μL) and 5 (1.8 mg/500 μL) mM. THR2 solutions were prepared in the same procedure as THR1 and concentrations of peptide were varied from 2 (0.7 mg/500 μL), 3 (1.1 mg/500 μL), 4 (1.4 mg/500 μL) and 5 (1.8 mg/500 μL) mM.

The results of colony forming are shown in figure 20. The RT112 cells that were treated with 5 mM of THR1, started to die on day 25 and completely dead on day 29 as same as the results from clonogenic assay 3. When the concentration of THR1 decreased, the ability of cells survival increased. From parallel study, THR2 did not show any significant or effect with bladder cells in all concentrations.

In conclusion, the suitable conditions of clonogenic assay were developed and optimized. At this point, we showed the reproducibility of anticancer activity of THR1 against RT112 cells.

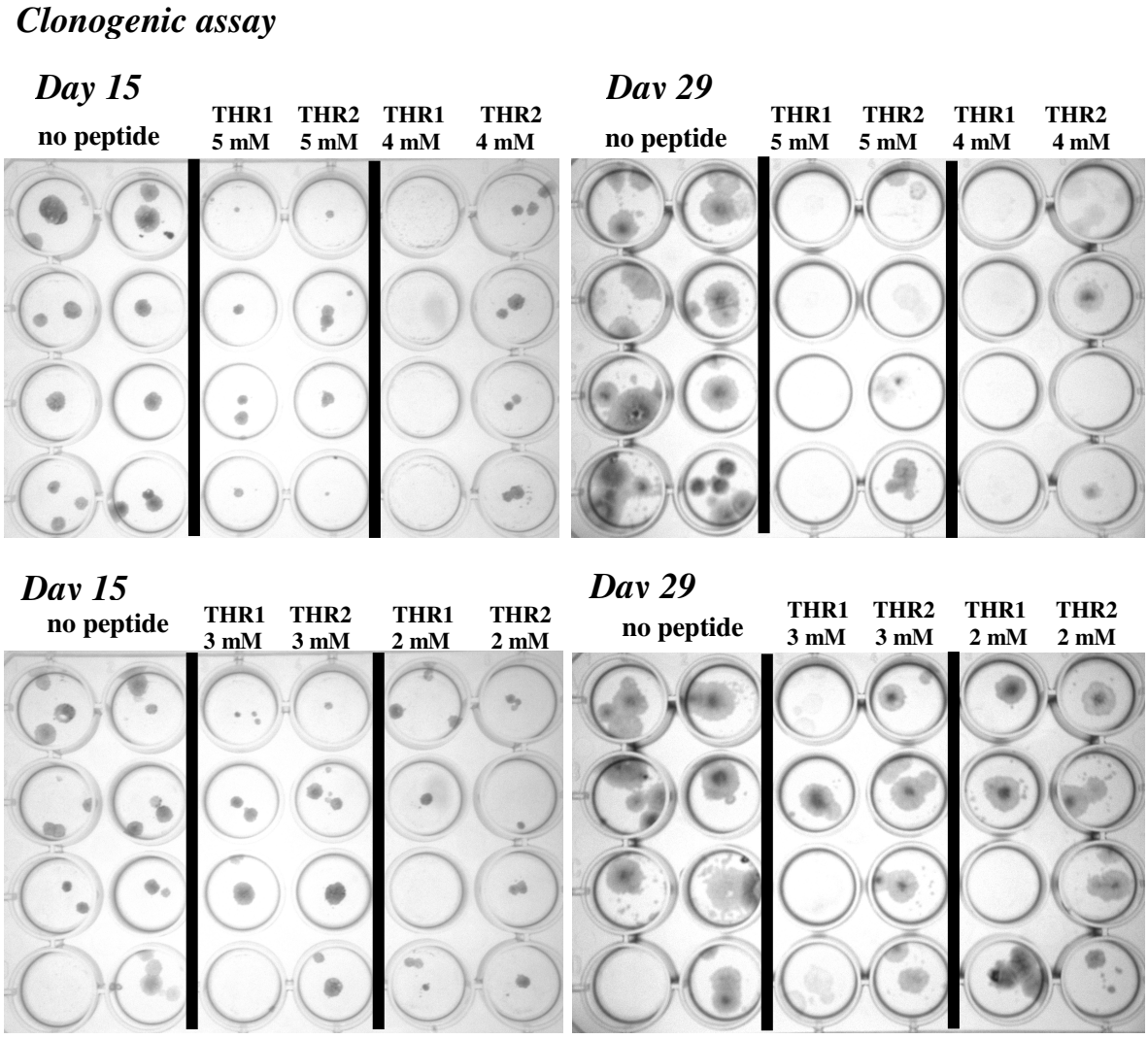


Figure 20: *Pictures of colony forming of RT112 bladder cancer cells on day 15 and 29. Bladder cells were trypsinized and seeded at 10 cells in each well. The cells were allowed to grow in medium for control experiment (without peptide, 500 µL of total volume). The anticancer activity was studied when cells exposed to THR1 and THR2. The concentration of both peptides was varied from 5, 4, 3, 2 mM (500 µL of total volume). This assay was performed in duplicate under the same conditions and kept at 37 ^{o}C in 5% CO_2 incubator. After 15 and 29 days, the cells were fixed with MeOH, stained with Giemsa's solution and visually examined under microscope for survival.*

CLONOGENIC ASSAY 5: an initial condition for MRC5-hTERT fibroblast cell lines.

MRC5-hTERT fibroblast cell line was chosen to study in this project because we really wanted to prove that our synthetic peptides showed excellent anticancer activity against cancer cell and did not have any significant effect with normal cells. The objectives of this assay were to optimize and find suitable cell density by vary number of normal cells suspension in each well.

Details: This assay that was done under the same conditions with RT112 cells was developed and optimized from previous assay. Because the nature of this fibroblast cells that could not form colonies, consequently the picture of colony forming in 24-well plate could not be taken.

As a result from the appearance of fibroblast cells (figure 21), we found that 10, 50 and 100 cells density in each well did not seem to be healthy on day 29. The number of cells was too small and not enough to treat with our peptides. While, if we plated 500 and 1000 cells density in each well, there were too many cells on day 29. Therefore, the suitable amount of cells suspension was 250 cells in each well.

In conclusion, from all biological assays development in this chapter, we decided to study the anticancer activity of our synthetic peptides by clonogenic assay against both of RT112 bladder cancer and MRC5-hTERT fibroblast cells. We found that RT112 10 cells/500 µL and MRC5-hTERT 250 cells/500 µL were suitable cells density to test with our synthetic peptides. The 24-well plate of were kept at 37 °C for 29 days in special incubator.

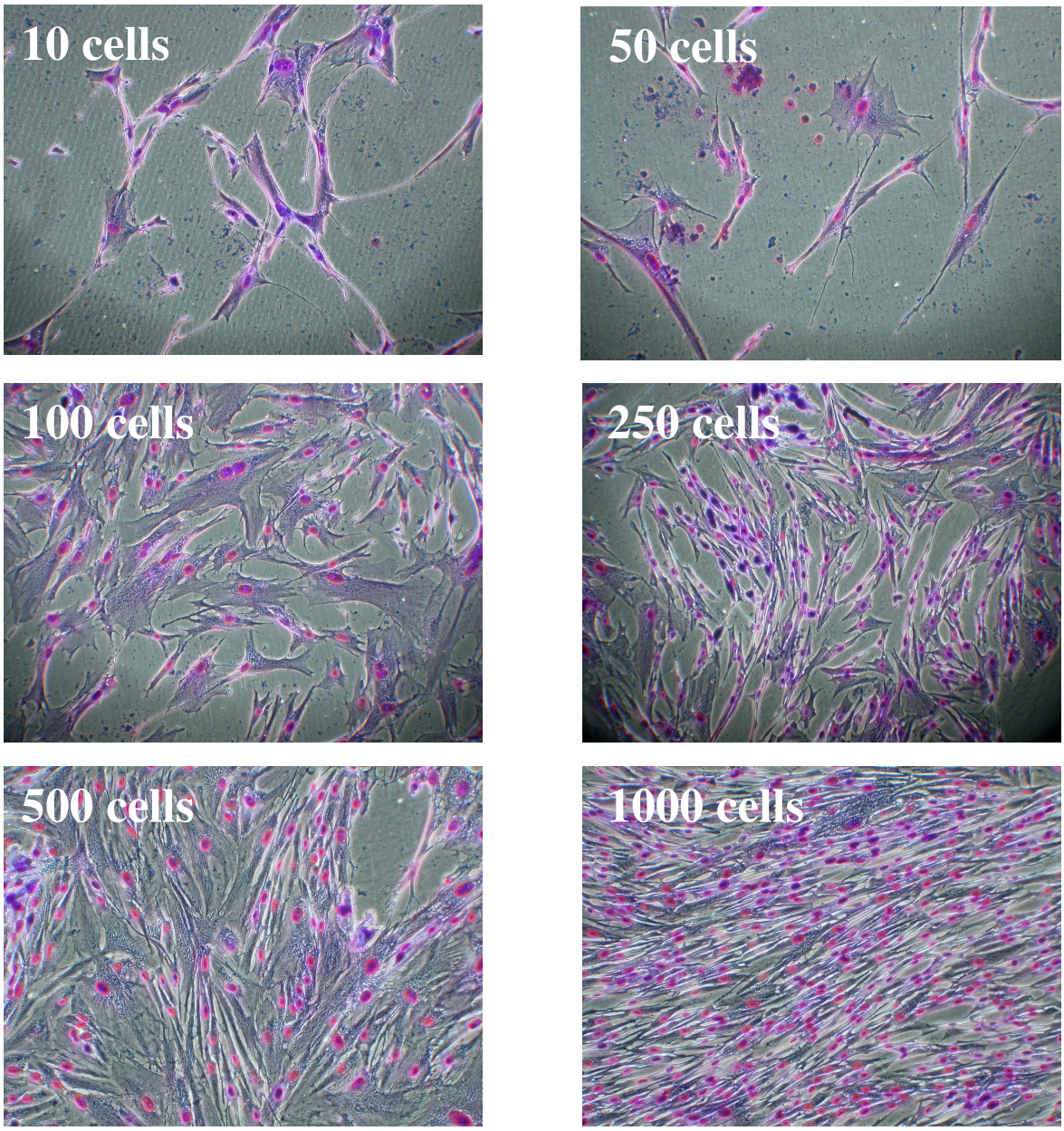
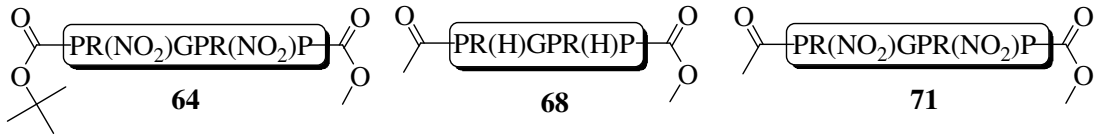


Figure 21: *Photomicrographs illustrating typical morphological appearances of MRC5-hTERT cells (without peptide) on day 29. The normal cells were trypsinized and seeded at 10, 50, 250, 500 and 1000 cells in each well. The cells were grown in DMEM medium for 29 days (without peptide, 500 µL of total volume). This assay was performed in duplicate under the same conditions and kept at 37 ^{o}C in 10% CO_2 incubator before staining with Giemsa's stain on day 29***.**

CHAPTER 8: STRUCTURE-ACTIVITY RELATIONSHIP (SAR) STUDY OF SYNTHETIC PEPTIDES BY CLONOGENIC ASSAY

8.1) SAR STUDY OF *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, 64 AND ITS SIDE CHAIN MODIFICATION PEPTIDES 68 AND 71.

Hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **64** was successfully synthesized by solution phase peptide synthesis. An attempt to synthesize compound **1** which had same structure with THR1, by side chain modification of compound **64** *via N*-Boc removal by strong acid, *N*-acetylation by acetic anhydride, amide formation at *C*-terminus and removal of nitro protecting group (-NO_2) of guanidine side chain by catalytic hydrogenolysis failed. Alternatively, a series of hexapeptide **64** was synthesized by partial side chain modification to obtain *N*-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe **68** and *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **71** (*N*-Ac group mimics with structure of THR1). The biological activities of *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **64** and SAR study of its analogue were investigated by clonogenic assay against RT112 bladder cancer and MRC5-hTERT fibroblast cells. The briefly structures of compound **64** and its analogue are shown in figure 22.



Scheme 22: The chemical structures of compound **64** and its side chain modification compounds **68** and **71**.

Details: Both of the cell lines were allowed to grow in 500 µL of tissue culture medium for control experiment. THR1 was prepared in medium (Ham's F-12 medium for RT112 bladder cancer cells and DMEM medium for MRC5-hTERT fibroblast cells) at 5 mM (1.8 mg/500 µL) and used as a positive control compound. The synthetic peptides **64** (2.2 mg/500 µL), **68** (2.0 mg/500 µL) and **71** (2.1 mg/500 µL) were prepared in the same procedure as described in section 6.3.1 at 5 mM. Plates were kept at 37 ºC, 5-10% CO_2 incubator, for 29 days.

Clonogenic assay on day 29

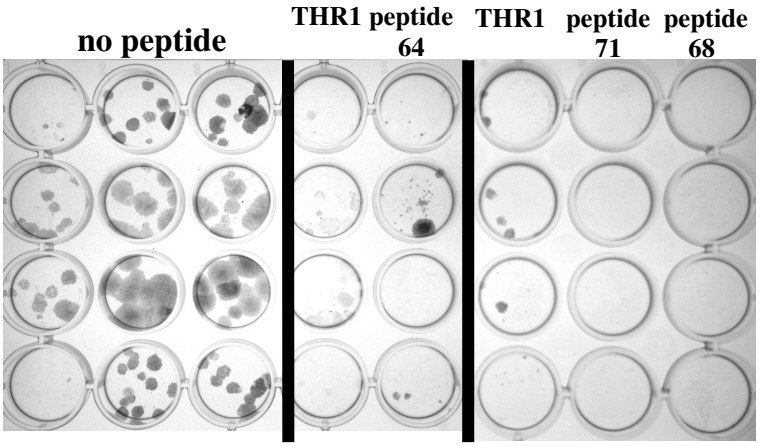


Figure 23: *Pictures of colony forming of RT112 bladder cancer cells on day 29. Control wells from only one plate were shown. Bladder cells were trypsinized and seeded at 10 cells in each well. The cells were grown in medium for control experiment (500 μL of final volume). The anticancer activity was studied when cells were exposed to 5 mM of peptide* ***64****,* ***68*** *and* ***71****. The results were compared with 5.0 mM of THR1 (5 mM = 1.8 mg/500 μL) for positive control experiment. This assay was performed in duplicate under the same conditions. Plates were kept at 37 ^{o}C in 5% CO_2 incubator before staining with Giemsa's stain on day 29.*

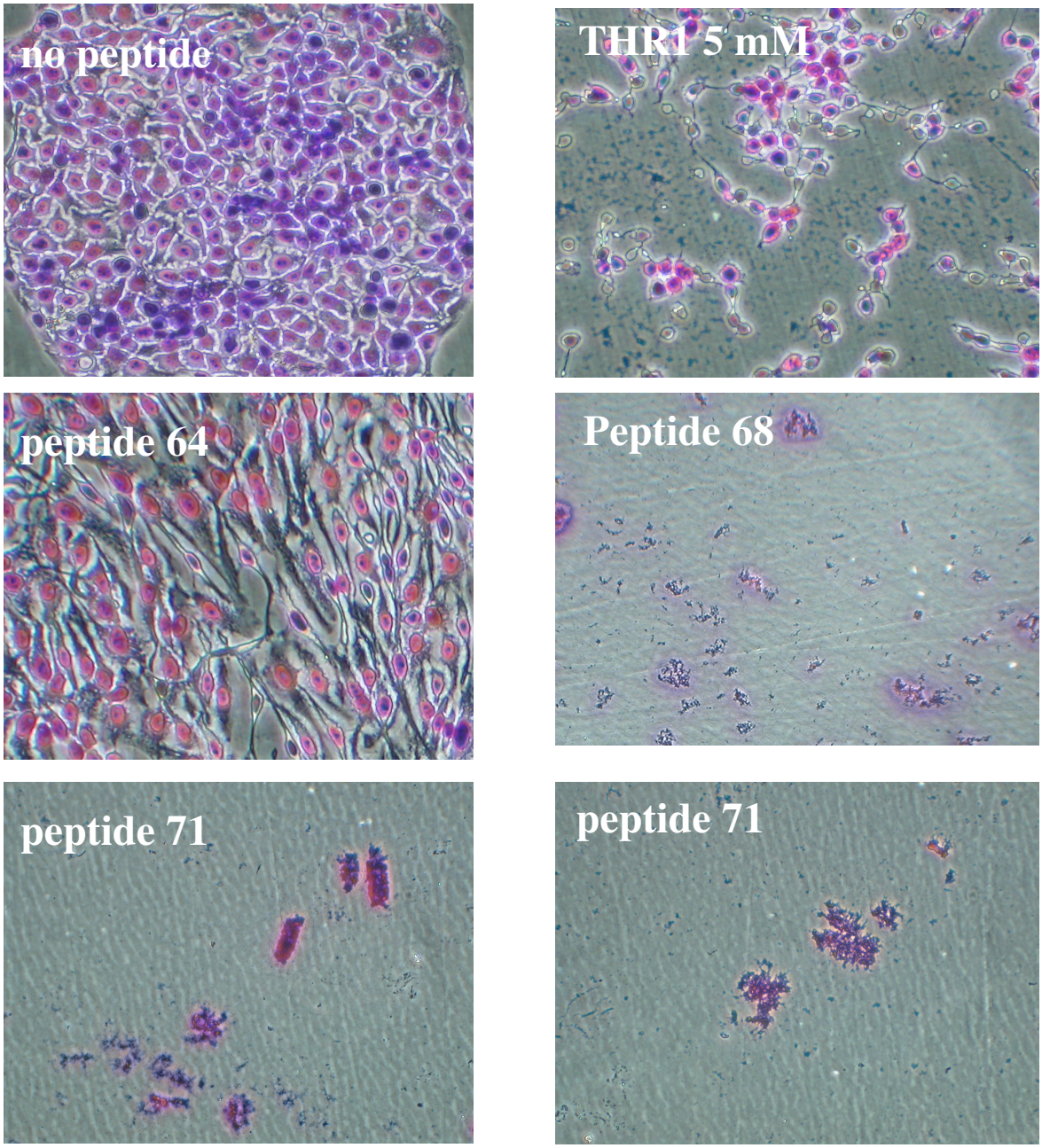


Figure 24: *Morphological appearances of RT112 cells exposured to 5 mM (500 µL) of synthetic hexapeptide **64**, **68** and **71** on day 29. The biological activities of these peptides were compared with 5 mM of THR1 (500 µL). Both of the peptides **68** and **71** could completely kill bladder cancer cells, while peptide **64** could not. There were some colonies left after exposed with peptide **64** for 29 days.*

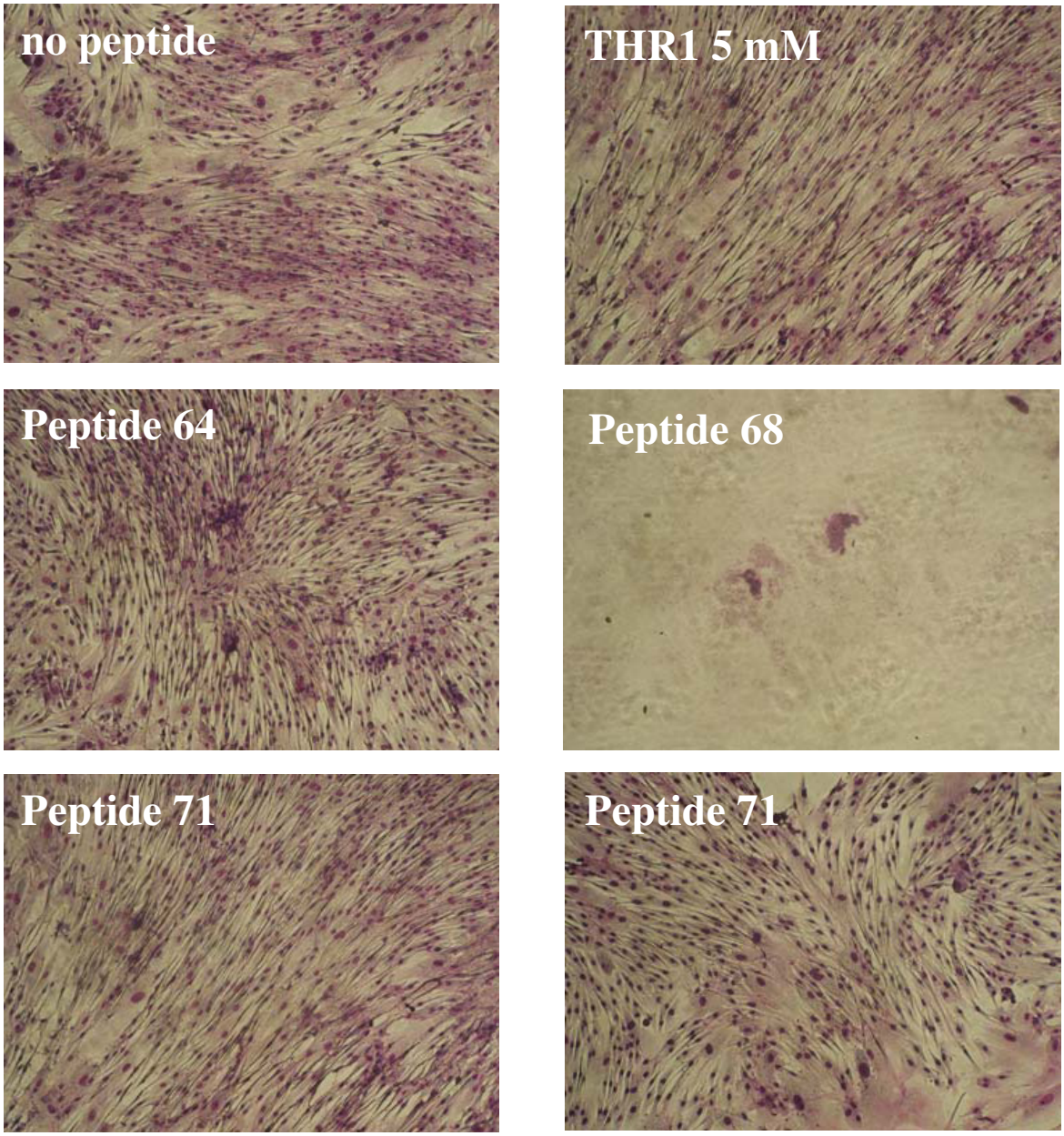


Figure 25: *Morphological appearances of MRC5-hTERT cells growing by expose to 5 mM (500 µL) of synthetic hexapeptide **64**, **68** and **71** on day 29. Normal cells were trypsinized and seeded at 250 cells suspension in DMEM medium into each well. The cells were grown in DMEM for control experiment (500 µL of final volume). The cells were exposed to 5 mM of peptide **64** (2.2 mg/500 µL), **68** (2.0 mg/500 µL) and **71** (2.1 mg/500 µL) and results compared with 5.0 mM of THR1 (5 mM = 1.8 mg/500 µL) for positive control experiment. This assay was performed in duplicate under the same conditions. Plates were kept at 37 ^{o}C in 10% CO_2 incubator before staining with Giemsa's stain on day 29.*

Results and discussion: the solution phase synthetic method was developed and optimized to synthesize a linear hexapeptide, *N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **64** and its partial side chain modified analogous **68**-**71**. The hexapeptides **68**-**71** were obtained for SAR study with different functional group at *N*- or *C*-terminus of peptide chain. Based on structure characterization, the hexapeptides **64**, **68** (*N*-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe) and **71** (*N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe) were chosen to treated RT112 bladder and fibroblast cells (5 mM). The anticancer activity of our synthetic hexapeptides was investigated by clonogenic assay and results compared with THR1 which was used as a positive control compound. The results on day 29 are shown in figure 23-25. The modified peptide **71** was obtained *N*-acetyl protecting group (mimic with structure of THR1) showed anticancer activity against bladder cancer cells. The hexapeptide **71** had completely killed cancer cells without killing normal fibroblast cell lines. The fully protected peptide **64** had slightly activity because there were some colonies of cancer cells left on day 29. While nitro-deprotected peptide **68** was a toxic peptide and killed both of cancer and normal cells.

8.2) SAR STUDY OF HEXAPEPTIDE, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, 71 AND ITS *L*-ALANINE SCANNING PEPTIDES 76, 83, 90, 95, 102 and 106 WHICH WERE SYNTHESIZED BY SOLUTION PHASE PEPTIDE SYNTHESIS.

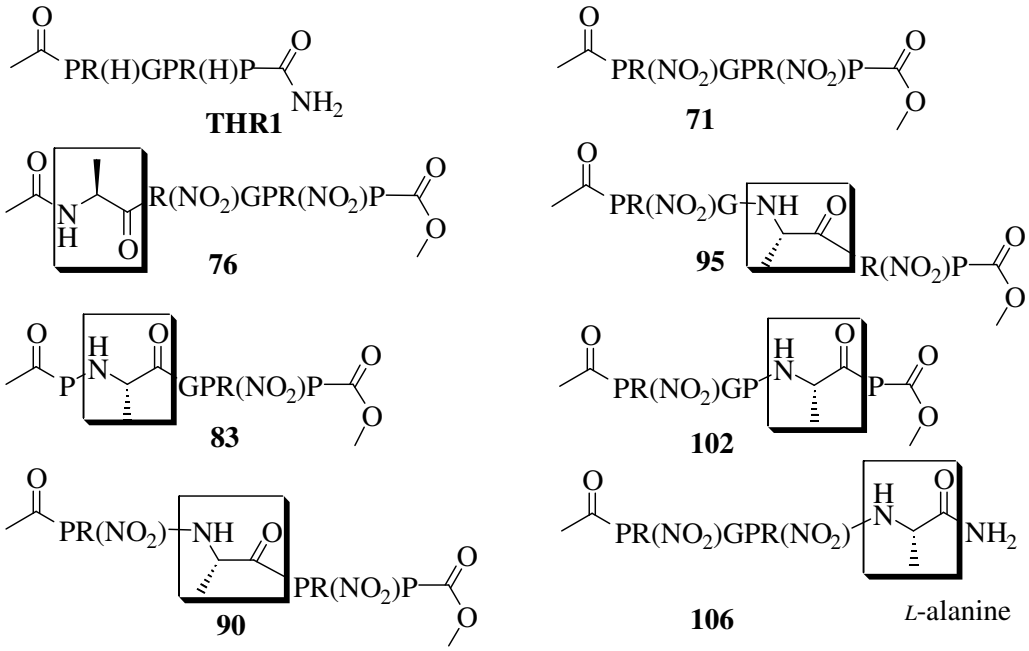
From the previous assay, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **71** that contained *N*-Ac protecting group, had the excellent of anticancer activity without killing normal cells. Therefore, to identify which position of amino acid in the parent structure **71** had the most effect upon the biological activity and to determine the most potent peptide structure in straight forward cancer cell killing assays, a series of compound **71** was synthesized by *L*-alanine (Ala) scanning method.

All *L*-Ala analogues were synthesized by solution phase peptide synthesis and *N*-terminus peptide was modified by *N*-Boc removal and *N*-acetal protected to obtain linear peptides *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **76**, *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **83**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **90**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **95**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe **102** and *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe **106** (as describe in chapter 2). The biological activities of *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **71** and its analogous were investigated by clonogenic assay against RT112 bladder cancer and MRC5-hTERT fibroblast cells. The chemical structures of all *L*-Ala scanning compounds are shown in figure 26.

Details: RT112 and MRC5-hTERT cells were allowed to grow in tissue culture medium 500 μL per each well dish for control experiment (without any peptide). The cells were exposed to 5 mM of THR1 solution for positive control experiment. Anticancer activities of our synthetic peptide **71** and its *L*-alanine analogous were investigated by clonogenic assay at 5 mM each peptide solution.

Peptide preparation and calculation: All peptide solutions were prepared in tissue culture medium (Ham's F-12 medium for RT112 bladder cancer cells and DMEM medium for MRC5-hTERT fibroblast cells) and added into each well of 24-well plate.

AcPRGPRP **71** = 2.1 mg/500 µL, AcARGPRP **76 =** 2.0 mg/500 µL, AcPAGPRP **83** = 1.7 mg/500 µL, AcPRAPRP **90** = 2.1 mg/500 µL, AcPRGARP **95** = 2.0 mg/500 µL, AcPRGPAP **102** = 1.7 mg/500 µL, AcPRGPRA **106** = 2.0 mg/500 µL , THR1 = 1.8 mg/500 µL.



Scheme 26: Chemical structures of all *L*-alanine scanning hexapeptides.

Clonogenic assay of RT112 on day 29

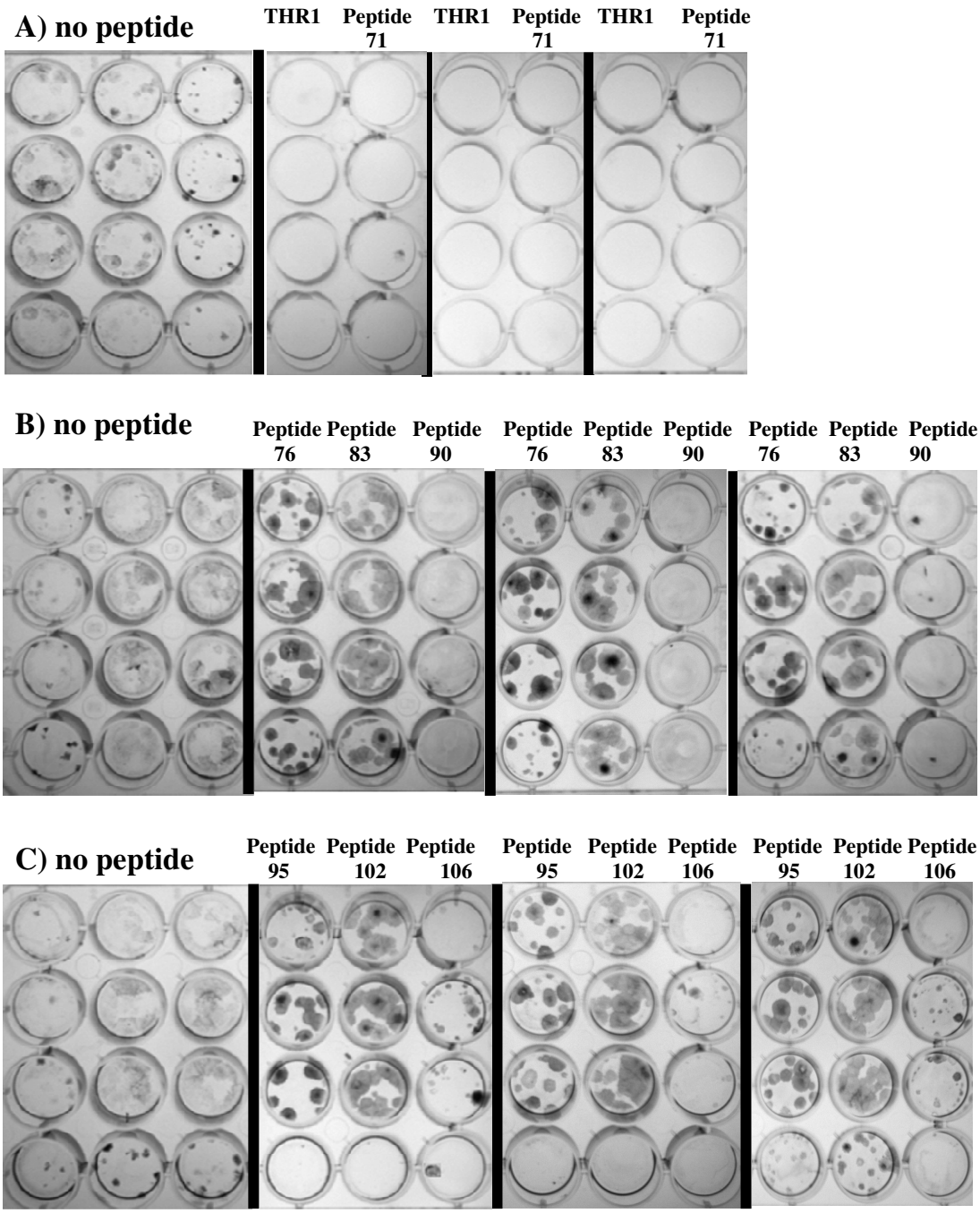


Figure 27: *Pictures of colony forming of RT112 cells on day 29. Control well from only one plate was shown. The cells were trypsinized and seeded at 10 cells in each well and grown in Ham's F-12 medium for control experiment (500 µL). Anticancer activity was studied when the cells were exposed to peptide* ***71*** *and its L-Ala compounds (5 mM). The results were compared with THR1 (5 mM). The assay was performed in triplicate plates under the same conditions. Plates were kept at 37 ^{o}C in 5% CO_2 before staining with Giemsa's stain on day 29.*

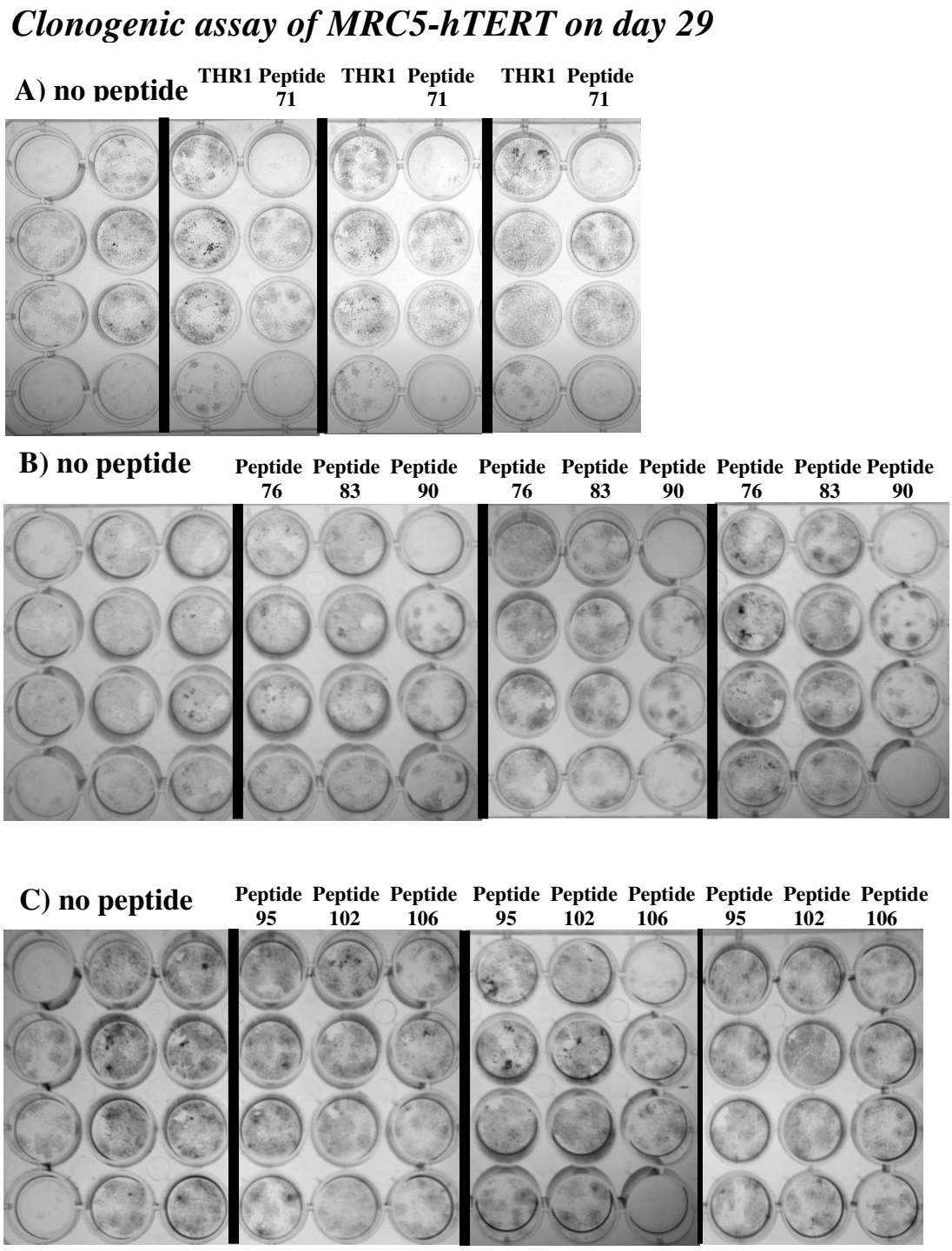


Figure 28: *Normal cells were trypsinized and seeded at 250 cells in each well. The cells were grown in DMEM for control experiment (500 µL). Control well from only one plate was shown. Anticancer activity was studied when the cells were exposed to peptide* ***71*** *and its L-alanine compounds (5 mM). The results were compared with THR1 (5 mM). This assay was repeated triplicate plates under the same conditions. Plates were kept at 37 ^{o}C in 10% CO_2 incubator before staining with Giemsa's stain on day 29.*

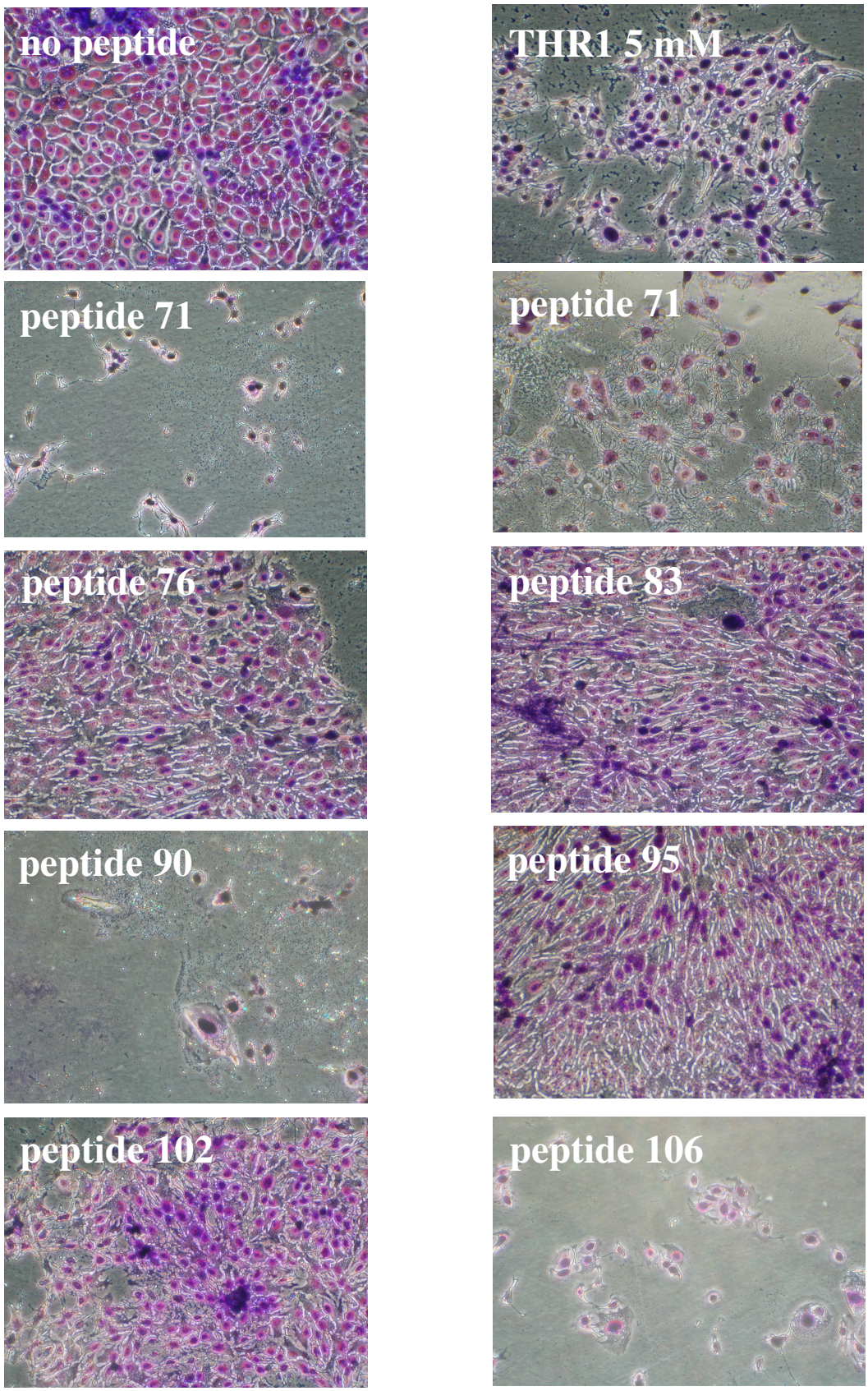


Figure 29: *Morphological appearances on day 29 of RT112 cells that were exposed with synthetic peptide **71** and its L-alanine scanning compounds at 5 mM (500 µL).*

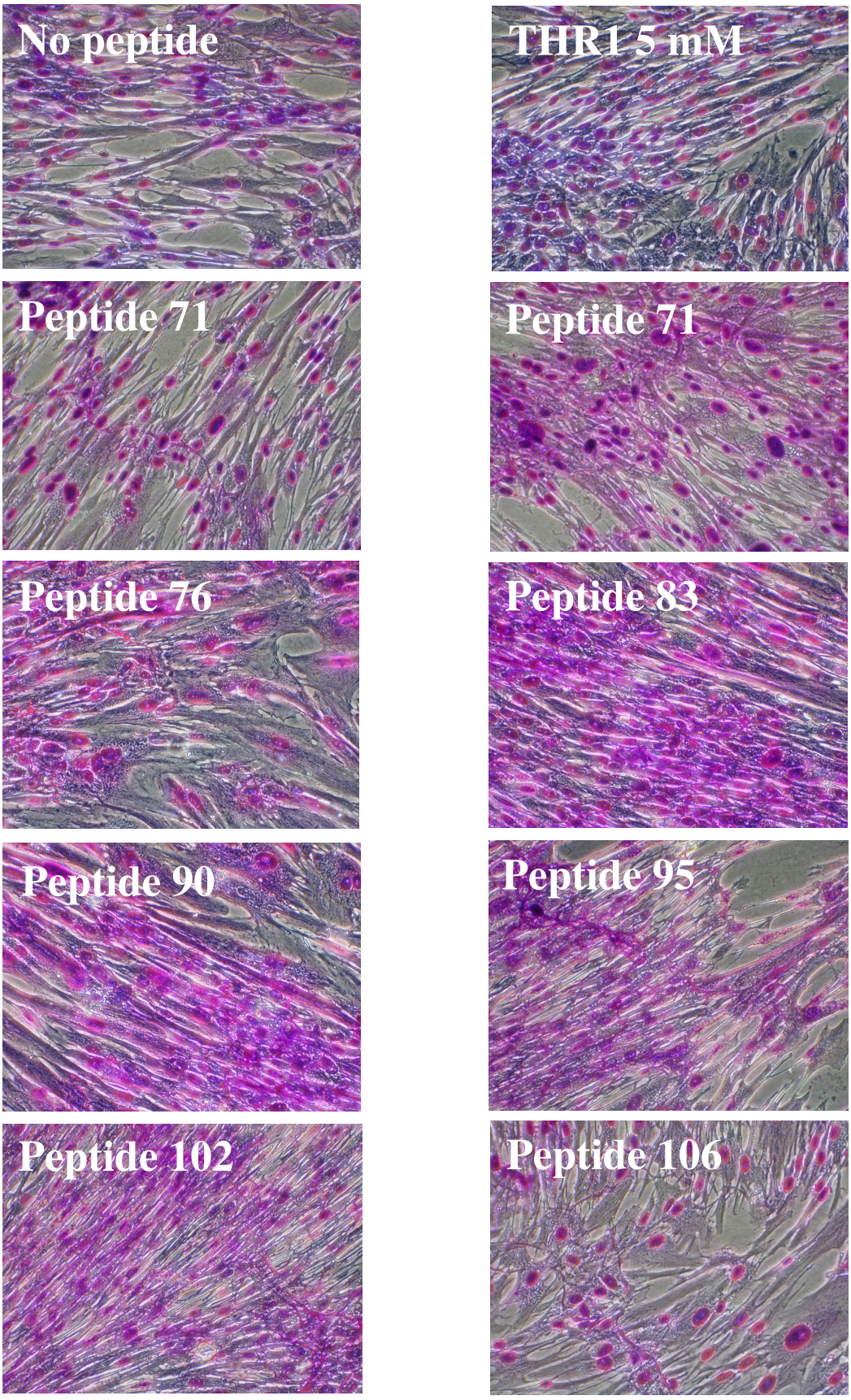


Figure 30: *Morphological appearances on day 29 of MRC5-hTERT cells that were exposed with synthetic peptide* ***71*** *and its L-alanine scanning compounds at 5 mM (500 µL).*

Results and discussion: A series of peptide **71** was synthesized by an *L*-alanine (Ala) scanning method to obtain compound **76**, **83**, **90**, **95, 102** and **106** which had *L*-Ala replaced at first, second, third, fourth, fifth and sixth amino acid residue of hexapeptide **71**. The anticancer activities of hexapeptide **71** and its *L*-Ala scanning compounds were investigated and studied by clonogenic assay. *N*-Ac peptide **71** and its *L*-Ala compounds were prepared at 5 mM and treated against both of bladder cancer and normal fibroblast cells. The results were compared with THR1 that was used as a positive control peptide (figure 27-30). The cells were exposed to peptide solution for 29 days. In this assay, the *N*-Ac peptide **71** was repeated to test with RT112 and MRC5-hTERT cell lines and results on day 29 confirmed reproducibility of peptide to kill cancer cells. In comparison with the anticancer activity of THR1, the peptide **71** showed the same results with THR1. A series of *L*-Ala peptides **76**, **83**, **95** and **102** could not kill both of RT112 bladder cancer and normal cells. On the other hand, peptides **90** and **106** were obtained anticancer activity against bladder cancer cells without killing normal cells.

8.3) SAR STUDY OF PARENT HEXAPEPTIDE 1, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, 1 AND ITS ANALOGUES 120-125 WHICH WERE SYNTHESIZED BY SOLID PHASE METHOD.

From solid phase peptide synthesis, we successfully synthesized compound **1** that had same structure with THR1 by solid phase method. As we knew from the previous biological assays, THR1 had excellent anticancer activity against RT112 cancer cells. The objectives of this work were to study anticancer activity of our synthetic peptide **1** by clonogenic assay against both of RT112 and MRC5-hTERT cell lines and compare the results with THR1. Furthermore, a series of *L*-alanine (Ala) scanning compounds **120**-**125** was synthesized by an analogous synthetic method. The aims of this work were to identify which position of amino acid in the parent structure **1** had effect upon the biological activity and to determine the most potent peptide structure for cancer cell killing assays.

Details: RT112 and MRC5-hTERT cells were allowed to grow in tissue culture medium 500 µL per each well for control experiment (without any peptide). The cells were exposed to 5 mM of THR1 for positive control experiment. Anticancer activities of our synthetic peptide **1** and its *L*-Ala analogous **120**-**125** were studied by clonogenic assay at 5 mM of each peptide solution.

Peptide preparation and calculation: All peptide solutions were prepared in tissue culture medium (Ham's F-12 medium for RT112 bladder cancer cells and DMEM medium for MRC5-hTERT fibroblast cells) and added into each well dish of 24-well plate.
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **1** = 1.8 mg/500 µL, *N*-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **120** = 1.7 mg/500 µL, *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **121** = 1.6 mg/500 µL, *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **122** = 1.8 mg/500 µL, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2 **123** = 1.7 mg/500 µL, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2 **124** = 1.6 mg/500 µL, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2 **125** = 1.7 mg/500 µL and THR1 = 1.8 mg/500 µL. The chemical structures for all of *L*-alanine scanning compounds are shown in scheme 64

Clonogenic assay of RT112 on day 29

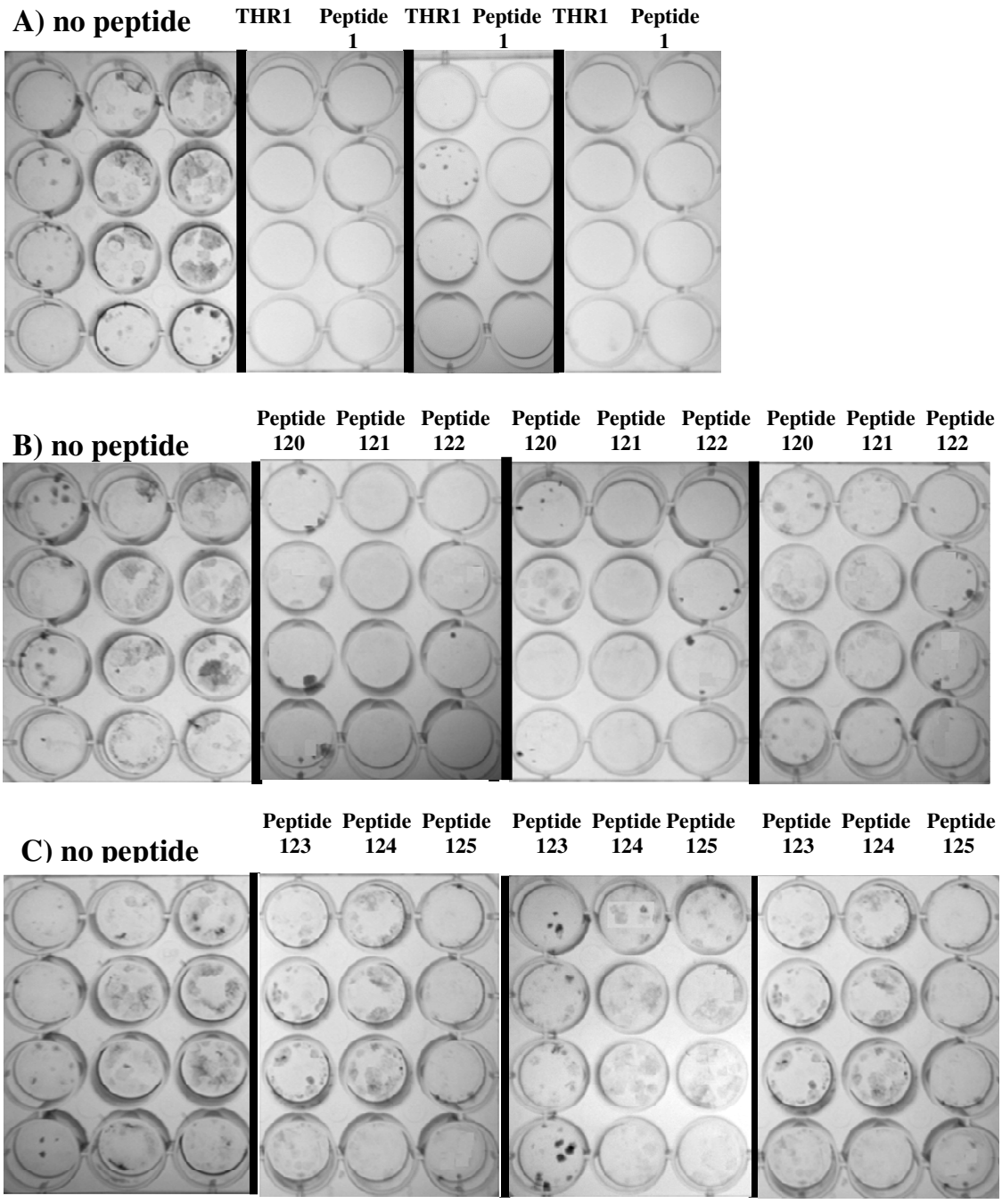


Figure 31: *Pictures of colony forming of RT112 cells on day 29. Control wells from only one plate were shown. The cells were trypsinized and seeded at 10 cells in each well and grown in Ham's F-12 medium for control experiment (500 µL). Anticancer activity was studied when the cells were exposed to peptide* ***1*** *and its* L*-Ala compounds (5 mM). The results were compared with THR1 (5 mM). The assay was repeated triplicate plates under the same conditions. Plates were kept at 37 ^{o}C in 5% CO_2 incubator before staining with Giemsa's stain on day 29.*

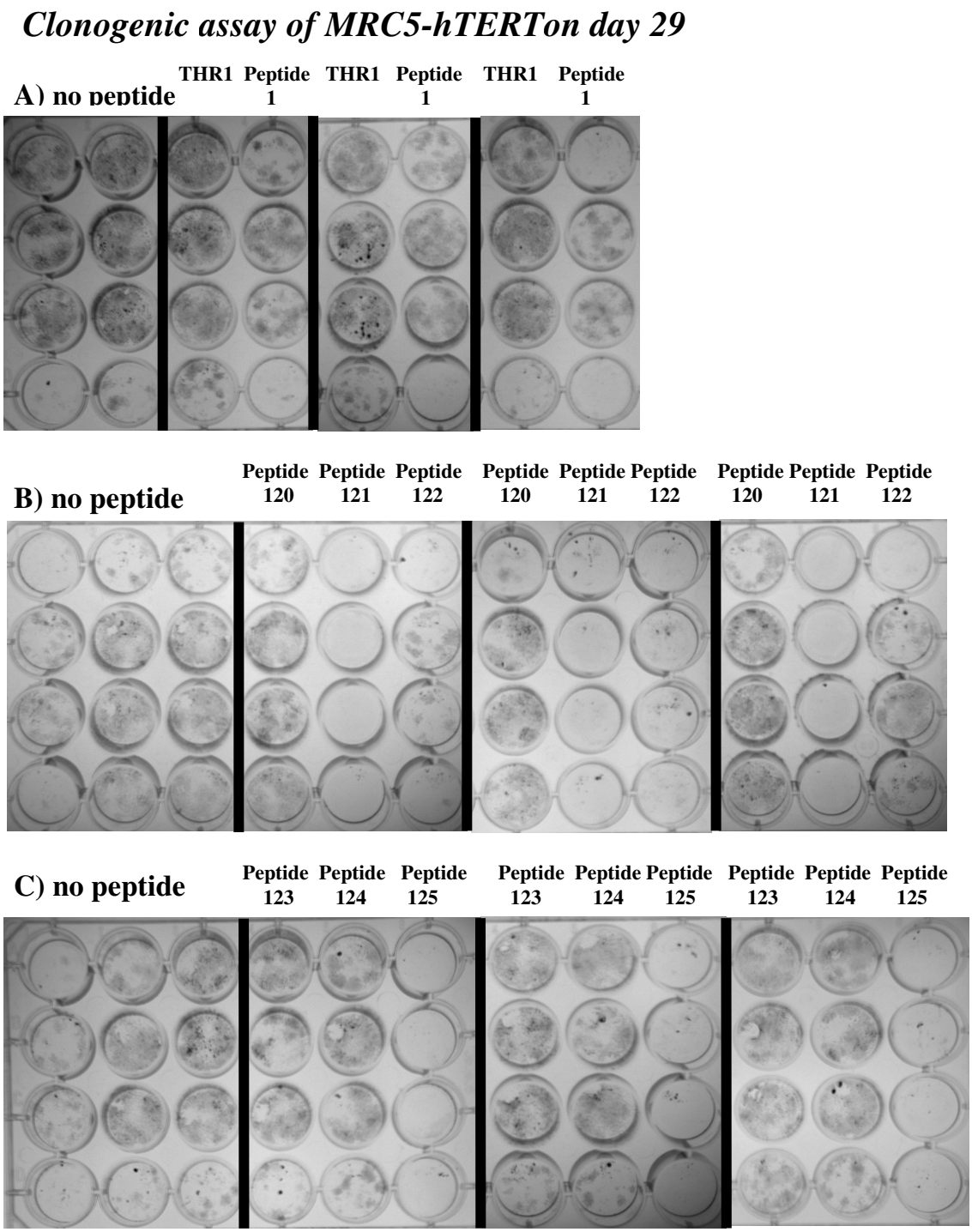


Figure 32: *Normal cells were trypsinized and seeded at 250 cells in each well and grown in DMEM for control experiment (500 μL). Anticancer activity was studied when the cells were exposed to peptide* ***1*** *and its L-alanine compounds (5 mM). The results were compared with THR1 (5 mM). This assay was repeated triplicate plates under the same conditions. Plates were kept at 37 ^{o}C in 10% CO_2 incubator before staining with Giemsa's stain on day 29. Control wells from only one plate were shown.*

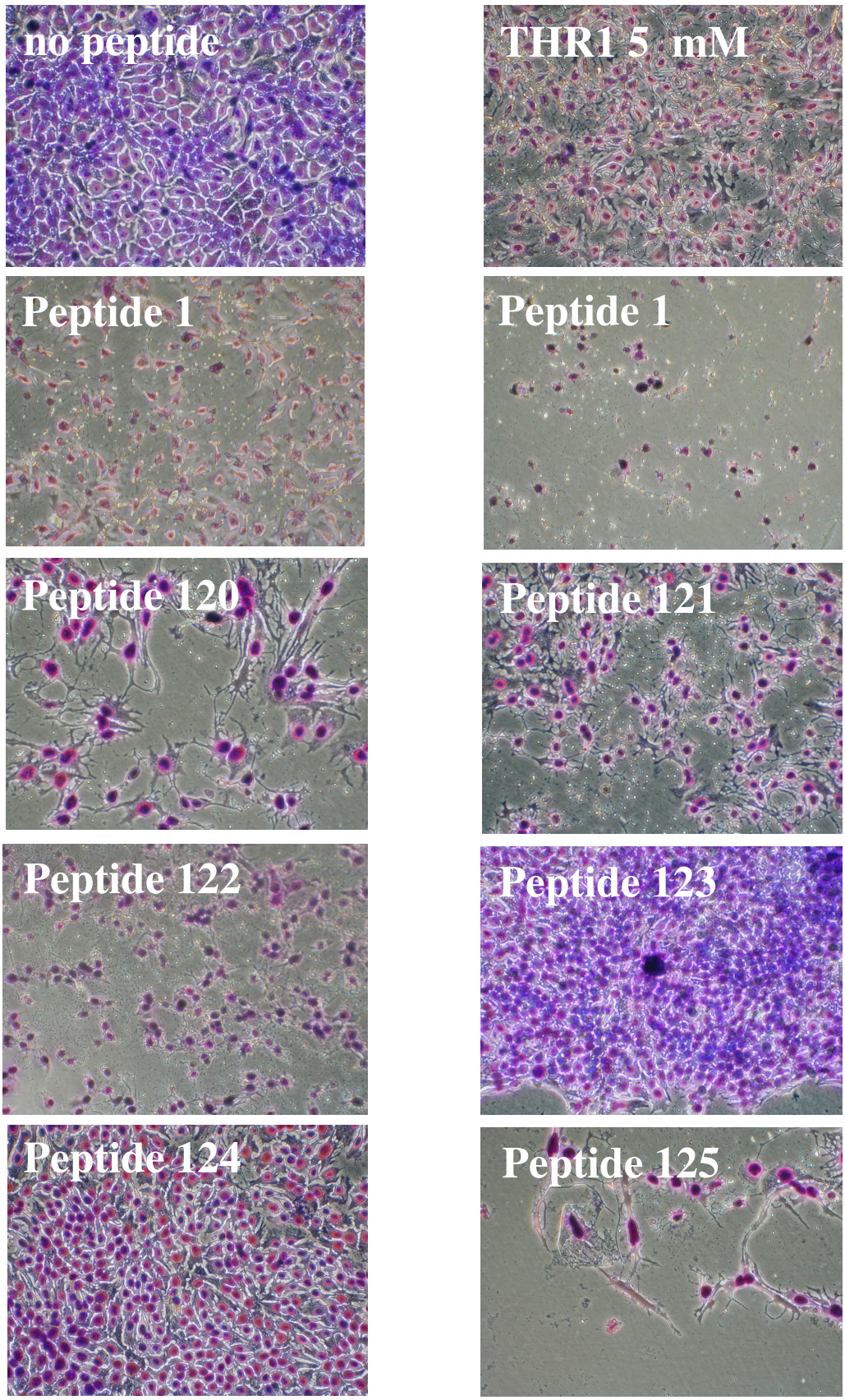


Figure 33: *Morphological appearances on day 29 of RT112 cells that were exposed to synthetic peptide **1** and its L-alanine scanning compounds at 5 mM (500 µL).*

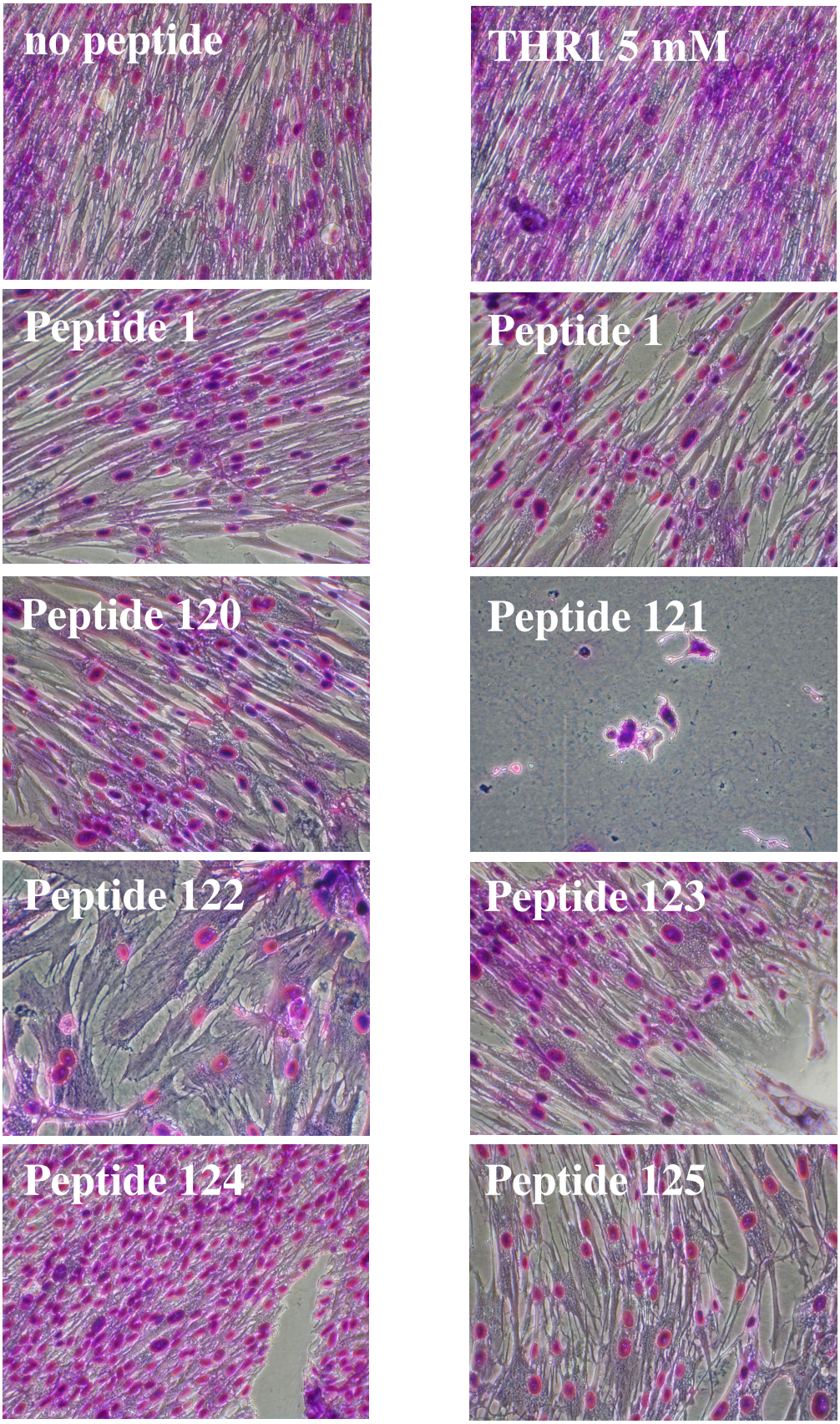
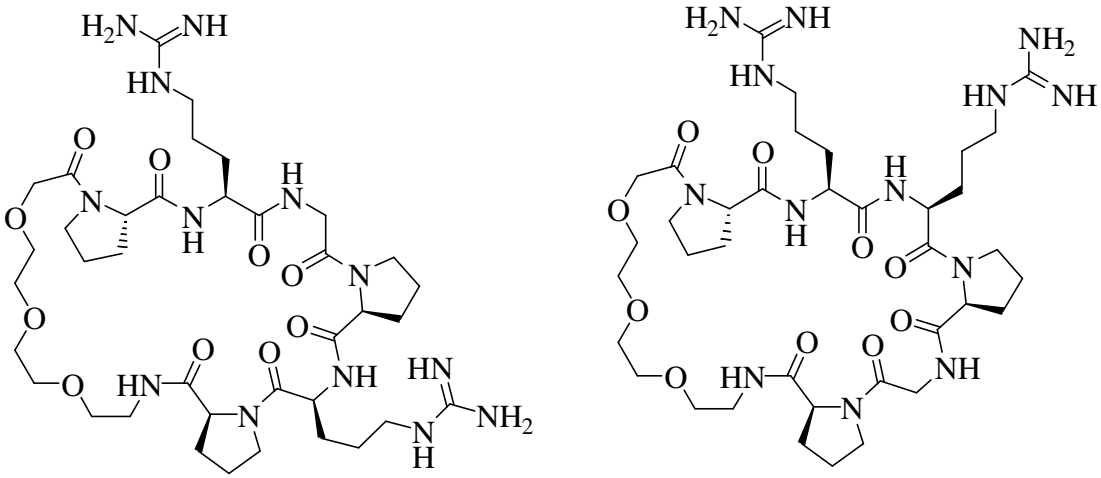


Figure 34: *Morphological appearances on day 29 of MRC5-hTERT cells that were exposed to synthetic peptide **1** and its L-alanine scanning compounds at 5 mM (500 µL).*

Results and discussion: The target hexapeptide **1** that could not be synthesized by solution phase method was successfully done by solid phase method. A series of *L*-Ala scanning compounds of hexapeptide **1** was also synthesized by an analogous synthetic method to obtain compounds **120**-**125**. The anticancer activity of these synthetic hexapeptides was investigated by clonogenic assay and compared results with THR1. As results showed in figure 31-34, we found that our target hexapeptide, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, **1** had better anticancer activity than THR1. The linear peptides **120** (*N*-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2), **122** (*N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) and **125** (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2) obtained anticancer activity against RT112 bladder cancer cells without killing normal cells. Peptide **121** (*N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) killed both of cell lines. Therefore, the hexapeptide **121** was toxic. While the peptide **123** (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2) and **124** (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2) could not kill both cell lines.

8.4) SAR STUDY OF CYCLIC PEPTIDES, *N,C*-PEG-tethered-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro 161 AND *N,C*-PEG-tethered-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro 168.

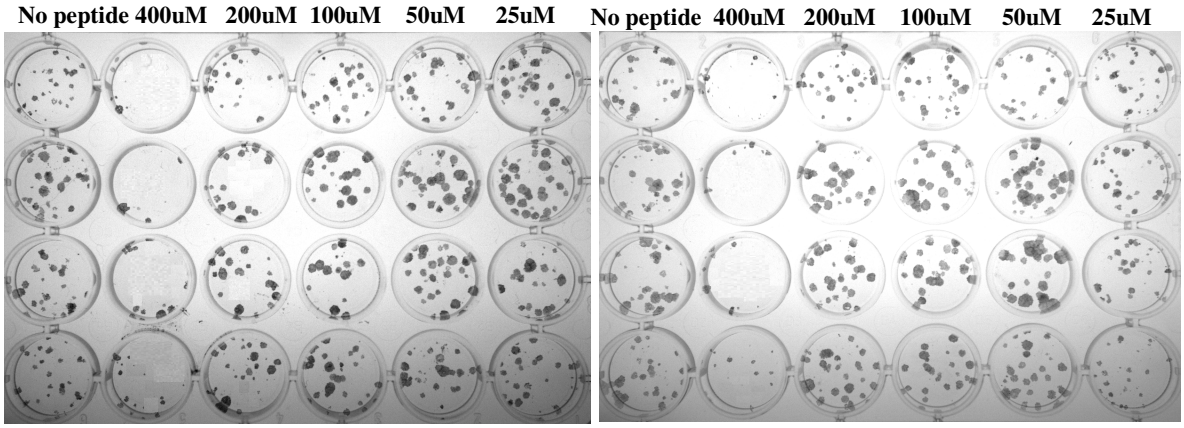
Cyclic peptides generally improved a biological property when compared to their linear counterparts. The cyclization may also be employed to constrain a bioactive peptide in its active conformation, thereby increasing its potency and/or specificity. The required linear hexapeptides **64** (*N*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe) and **150** (*N*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe) were synthesized by solution phase method (as described in chapter 2 and 4, respectively) and used as precursors for cyclic peptide synthesis. The modified polyethylene glycol **155** was synthesized and coupled with both *N*- and *C*-terminus of linear peptides **64** and **150** to obtain cyclic molecules **161** and **168**, respectively. Chemical structures of these cyclic peptides are shown in figure 36.



161 **168**

Figure 36: Chemical structures of cyclic peptides **161** and **168**.

A) Clonogenic assay on day 12 of cyclic peptide 161



B) Clonogenic assay on day 12 of cyclic peptide 168

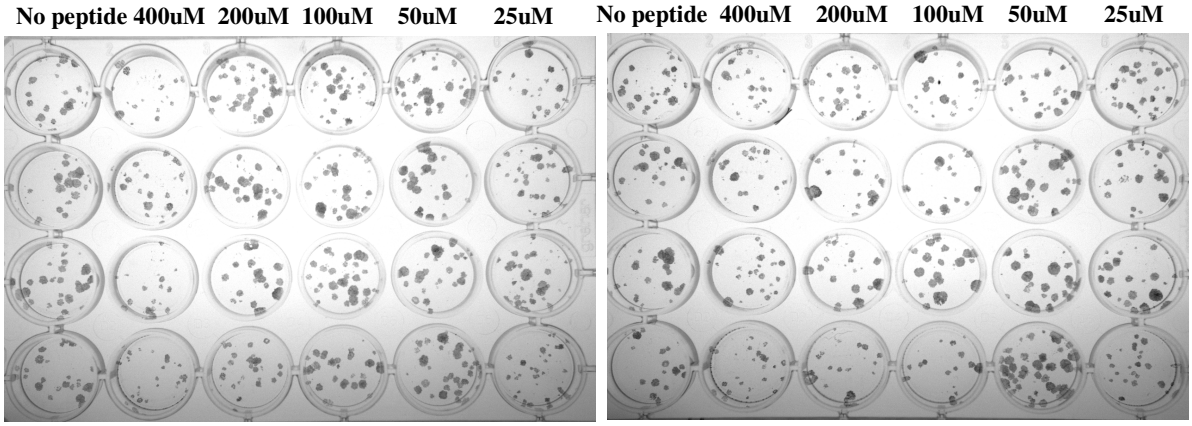


Figure 36: *Pictures of colony forming of RT112 Bladder cells on day 12. Cells were trypsinized and seeded 20 cells in each well. The cells were grown in 500 µL of Ham's F-12 for control experiment (without peptide). Anticancer activity of [cyclo]-PRGPRP* ***161*** *(figure 36A) and [cyclo]-PRRPGP* ***168*** *(figure 36B) was studied by clonogenic assay. The cyclic peptide solutions* ***161*** *and* ***168*** *were prepared in Ham's F-12 medium and concentration of each peptide was varied from 400, 200, 100, 50, 25 µM. The peptide solutions were added into each well (500 µL of total volume). This assay was performed in duplicate under the same conditions. Plates were kept at 37 °C in 5% CO_2 incubator before staining with Giemsa's stain on day 12.*

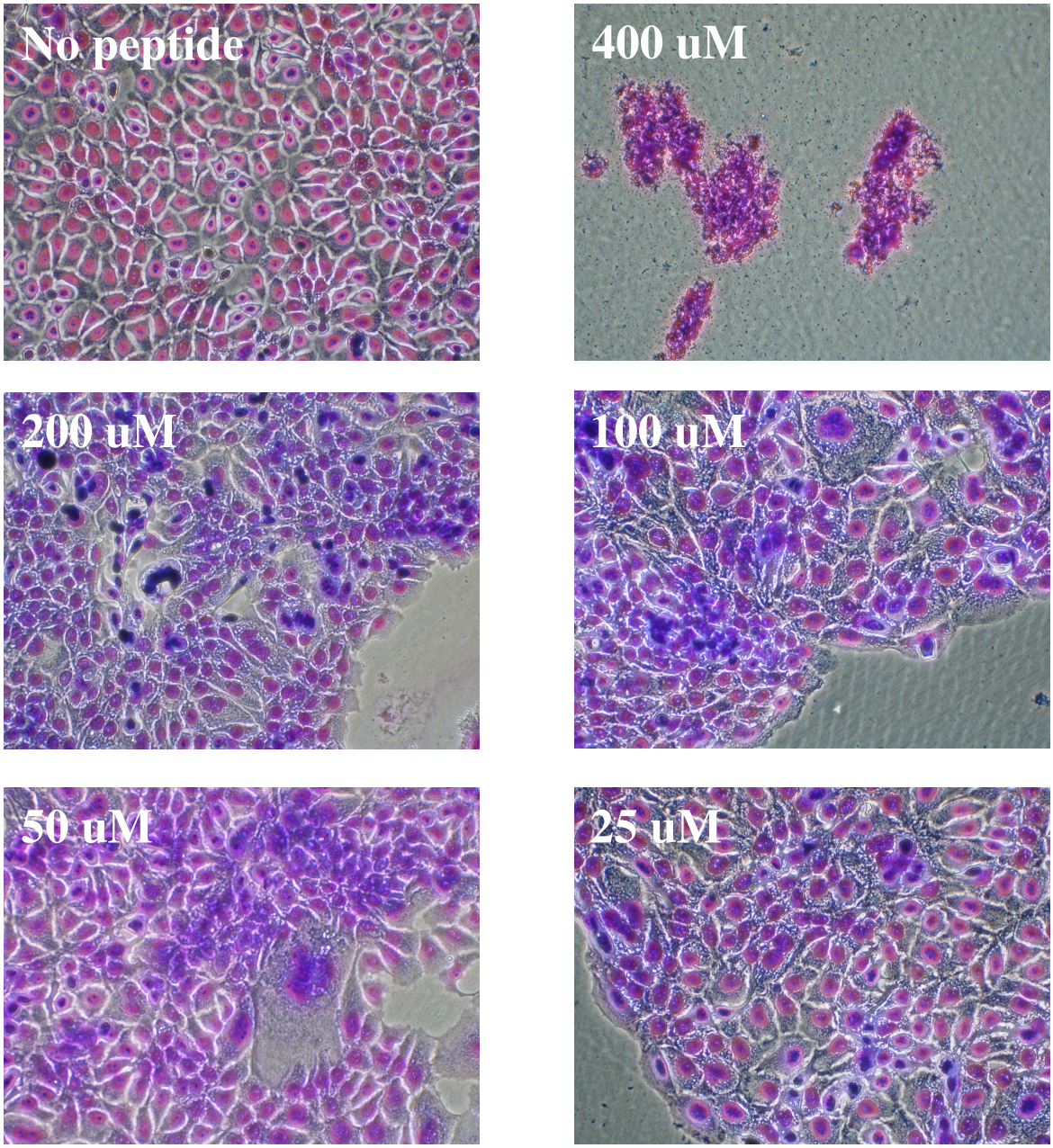


Figure 37: *Morphological appearances of RT112 cells growing in vitro following exposure to cyclic peptide* ***161*** *([cyclo]-PRGPRP) on day 12. The concentration of peptide solution was varied from 400, 200, 100, 50 and 25 μM and results compared with control experiment (without peptide).*

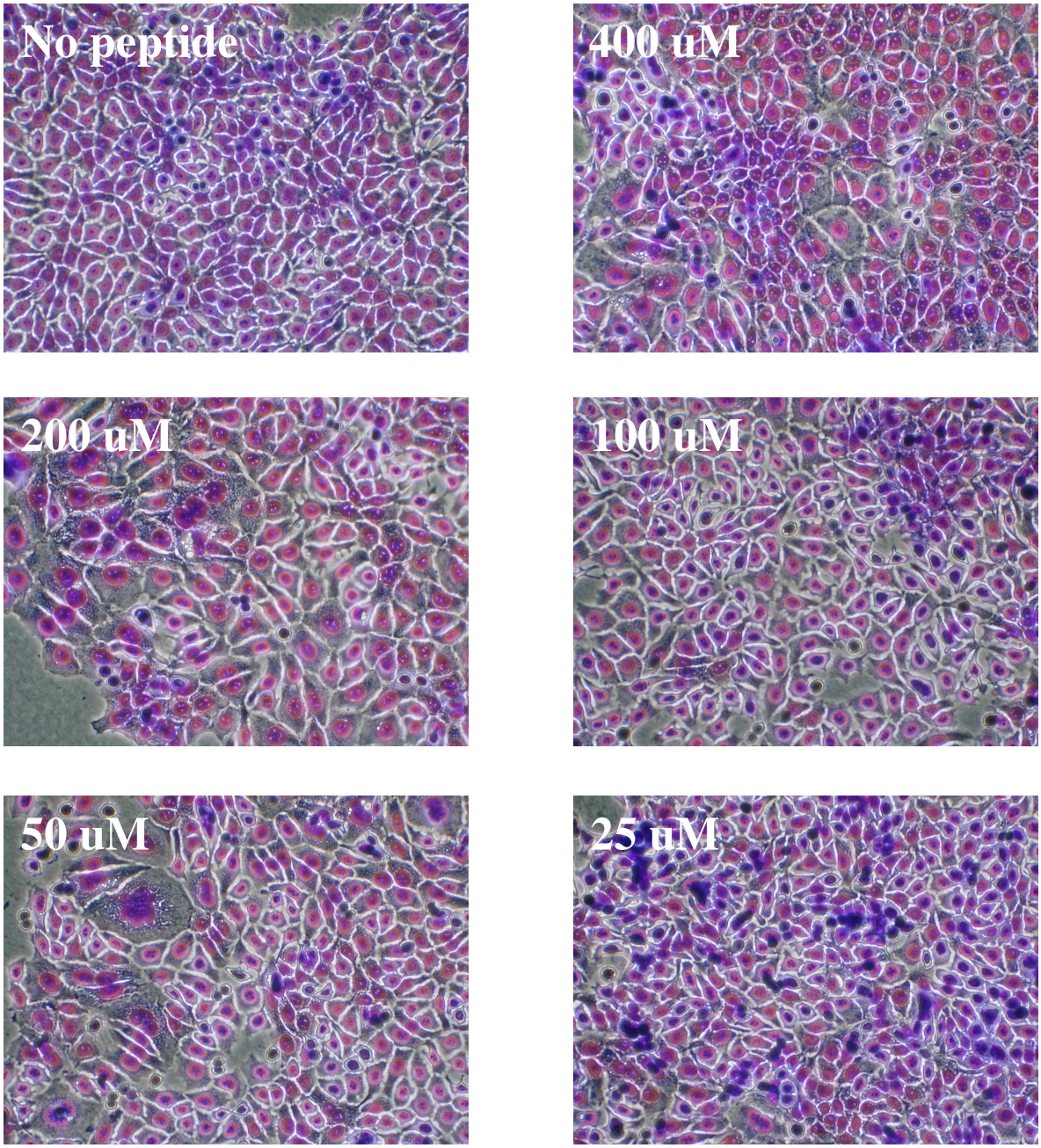
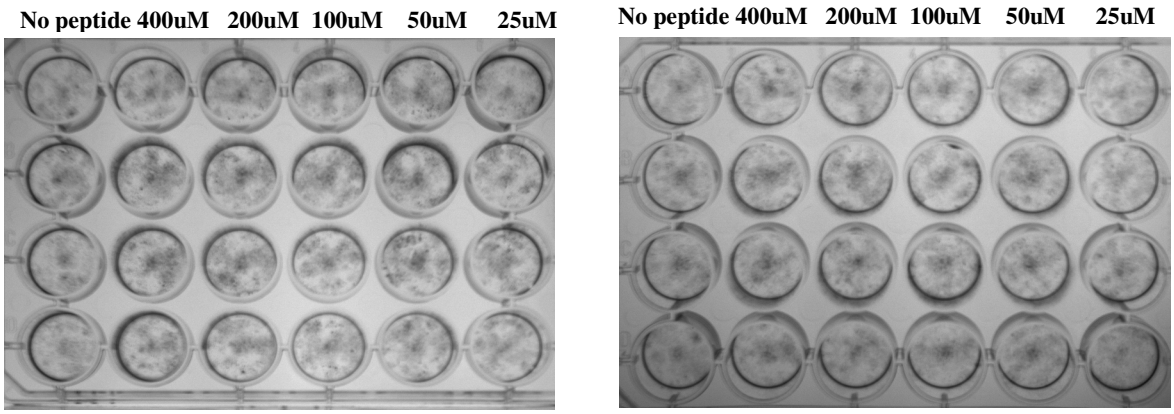


Figure 38: *Morphological appearances of cell death in RT112 cells growing in vitro following exposure to cyclic peptide* ***168*** *([cyclo]-PRRPGP) on day 12. The concentration of peptide solution was varied from 400, 200, 100, 50 and 25 μM and results compared with control experiment (without peptide).*

A) Clonogenic assay on day 12 of cyclic peptide 161



B) Clonogenic assay on day 12 of cyclic peptide 168

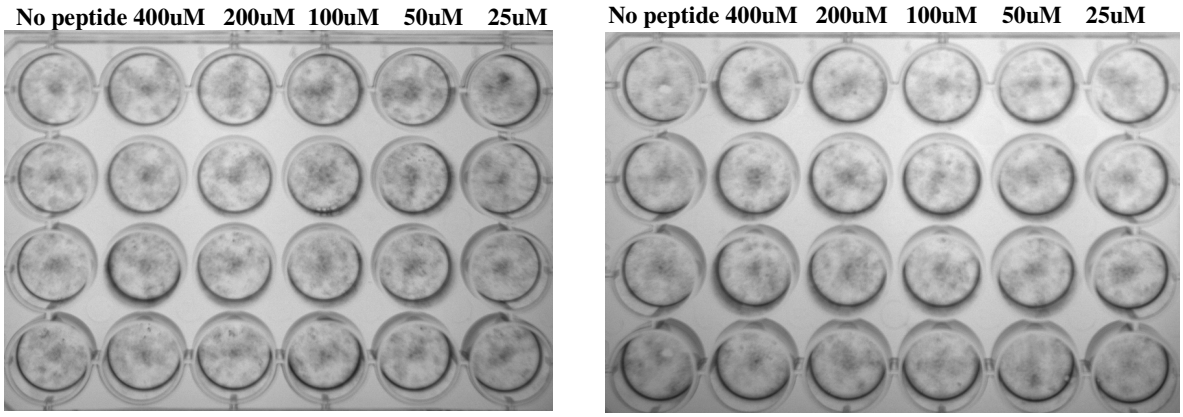


Figure 39: *MRC5-hTERT fibroblast cells were trypsinized and seeded at 250 cells in each well. The cells were grown in 500 μL of DMEM medium for control experiment (without peptide). Anticancer activity of [cyclo]-PRGPRP* ***161*** *(figure 39A) and [cyclo]-PRRPGP* ***168*** *(figure 39B) was studied by clonogenic assay. The cyclic peptide solutions* ***161*** *and* ***168*** *were prepared in DMEM medium. The concentration of each peptide was varied from 400, 200, 100, 50, 25 μM. The peptide solutions were added into each well (500 μL of total volume). This assay was performed in duplicate under the same conditions. Plates were kept at 37 °C in 10% CO_2 incubator before staining with Giemsa's stain on day 12.*

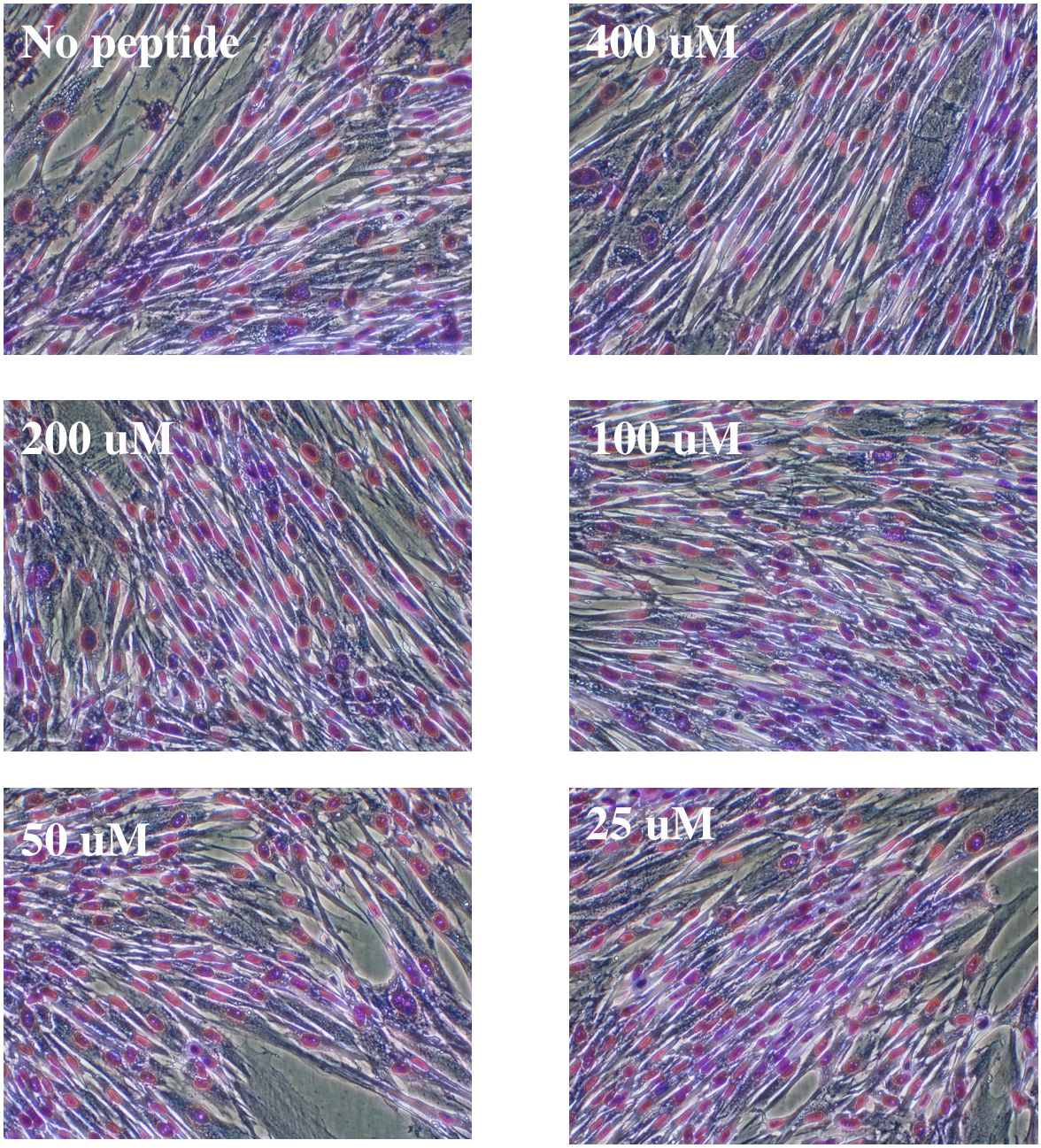


Figure 40: *Morphological appearances of MRC5-hTERT cells growing exposure to cyclic peptide **161** ([cyclo]-PRGPRP) with indicated concentration on day 12. The concentration of peptide solution was varied from 400, 200, 100, 50 and 25 μM and results compared with control experiment (without peptide).*

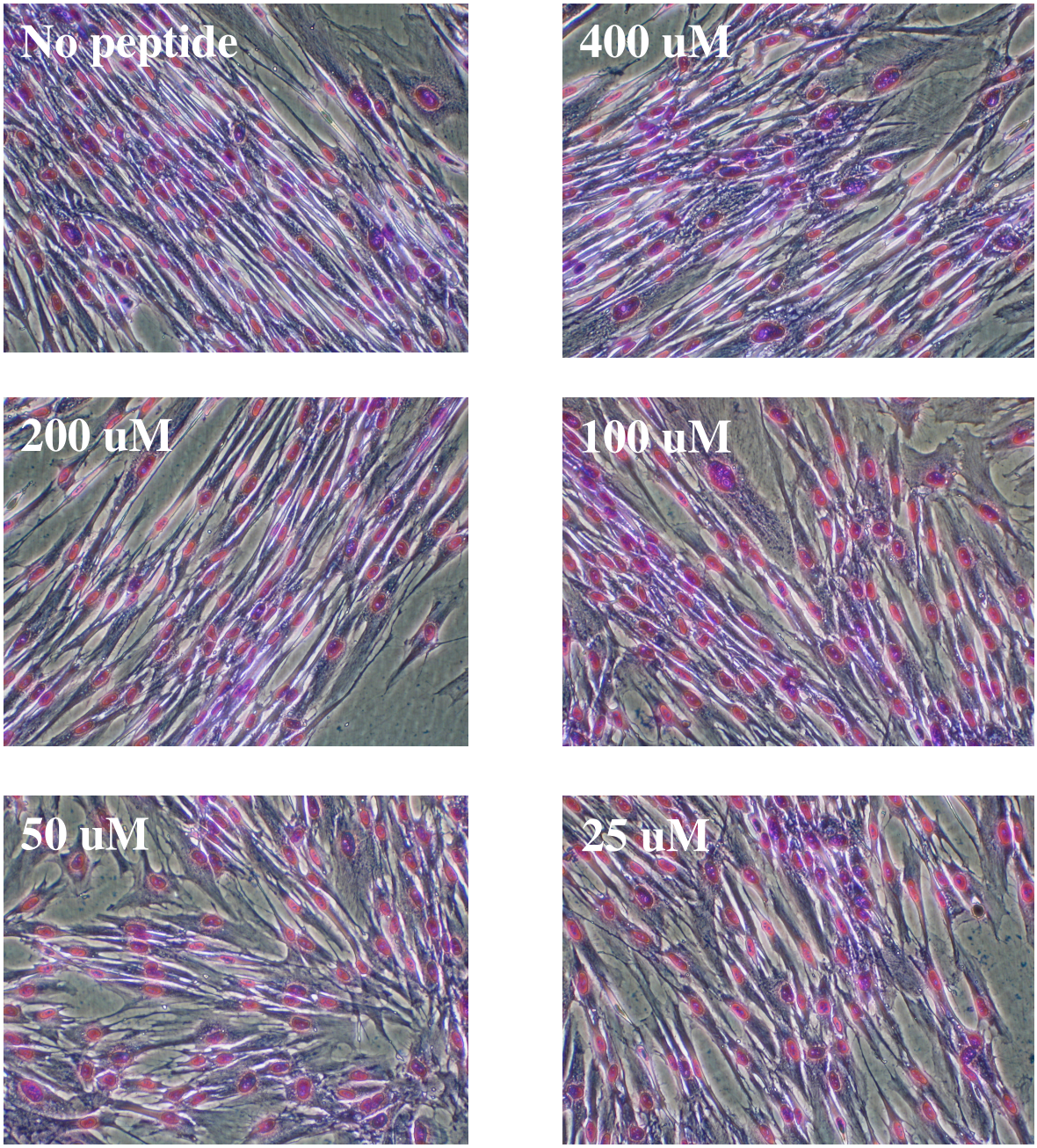


Figure 41: *Morphological appearances of MRC5-hTERT cells growing exposure to cyclic peptide **168** ([cyclo]-PRRPRP) with indicated concentration on day 12. The concentration of peptide solution was varied from 400, 200, 100, 50 and 25 μM and results compared with control experiment (without peptide).*

As results on day 12, we found that peptide **161** at 400 μM displayed an excellent anticancer activity against RT112 bladder cancer cells without killing normal cells. The activity of cyclic peptide **161** was decreased when concentration of peptide solution was decreased from 200 μM, 100 μM, 50 μM and 25 μM, respectively. In parallel study with

cyclic peptide **168** which was used as negative controlled peptide, could not kill both of bladder cancer and fibroblast cells (figure 36-41).

8.5) CONCLUSIONS

In conclusion, firstly, the target hexapeptide **1**, *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, had the same structure with THR1 showed anticancer activity against RT112 bladder cancer cells without killing MRC5-hTERT cells. Secondly, partial side chain modification, peptide **71**, *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, completely killed bladder cancer cells without killing normal fibroblast cells. Therefore, *C*-terminus protecting group (-OMe) and guanidine protecting group at *L*-Arg side chain (-NO_2) of peptide chain had not been involved with cytotoxic activity of peptide chain. Thirdly, for *L*-Ala scanning compounds, if the *L*-Ala was substituted at each position of *L*-amino acid residue in peptide chain [*L*-Pro-*L*-Arg(*R)-*L*-Gly-*L*-Pro-*L*-Arg(*R)-*L*-Pro] and would decrease cytotoxicity of peptide. Therefore, *L*-amino acid residue in the peptide chain had been involved with anticancer activity. As results in section 8.2, *L*-Ala substitution in peptides at position 1, 2, 4 and 5 did not have cytotoxic activity against RT112 cells. We assumed that Pro-1, Arg-2, Pro-4, and Arg-5 were the essential *L*-amino acid residues for anticancer activity. As results in section 8.3, peptides were synthesized by solid phase method. We found that peptide **123** and **124** did not have cytotoxic activity aginst RT112 bladder cancer cells. We concluded that Pro-4 and Arg-5 strongly influenced with anticancer activity of peptide. Finally, for cyclic peptide synthesis, we found that cyclic peptide **161** (400 μM) completely killed cancer cell and had better activity than straight chain peptide. The improvement of activity in cyclic peptide might be due to rigidity of molecule especially an active part [*L*-Pro-*L*-Arg(H)-Gly -*L*-Pro-*L*-Arg(H)-*L*-Pro]. It was also demonstrated that the cytotoxic activity of peptides strongly influenced by the sequences of amino acid residues in peptide chain.

On the basis of our structural investigations, the structure-activity relationships of the bioactive peptides can be summarized as follows.

Table 1: Conclusion of biological assay of all peptides

PEPTIDES	RT112	MRC5-hTERT
SOLUTION PHASE PEPTIDE SYNTHESIS		
THR1 (positive controlled compound)	**--**	**++**
N-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **64**	**++**	**++**
N-Boc-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-OMe, **68**	**--**	**--**
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **71**	**--**	**++**
N-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **76**	++	++
N-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **83**	++	++
N-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe, **90**	--	++
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe, **95**	++	++
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe, **102**	++	++
N-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe, **106**	--	++
SOLID PHASE PEPTIDE SYNTHESIS		
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **1**	--	++
N-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, **120**	--	++
N-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, **121**	--	--
N-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2, **122**	--	++
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2, **123**	++	++
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2, **124**	++	++
N-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2, **125**	--	++
CYCLIC PEPTIDES		
Cyclo-PEG- Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro **161** (**)	--	++
Cyclo-PEG- Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro **168**	++	++

*(*R) = -H for solid phase peptide synthesis or -NO_2 for solution phase peptide synthesis.*

(**) *cyclic peptide **161** showed anticancer activity at 400 μM.*

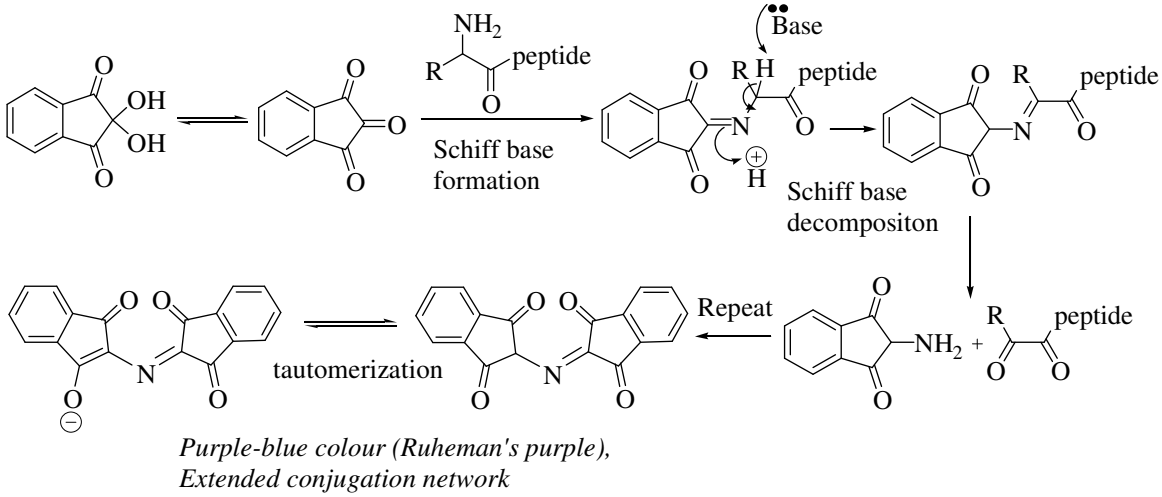
++ = *cells were survived*

-- = *cells were dead*

APPENDIX:

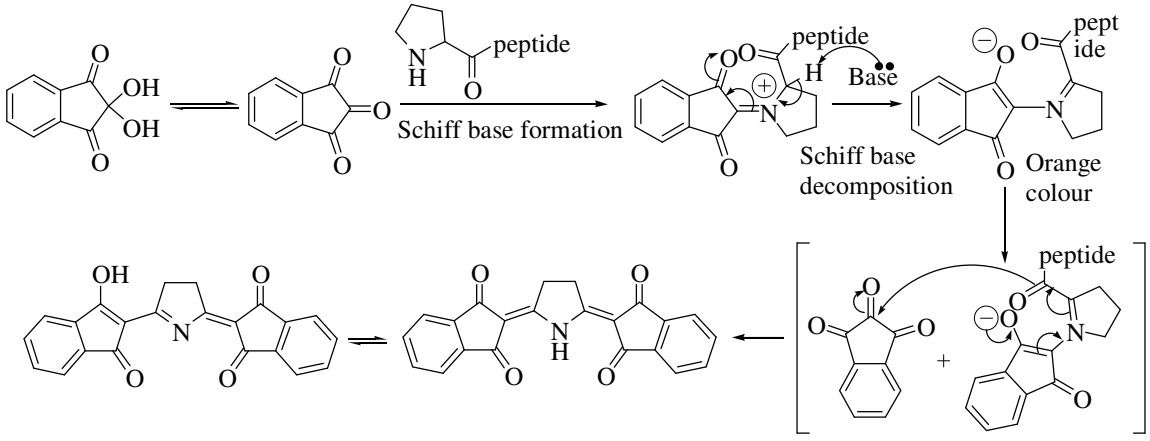
Appendix 1: The mechanism of primary and secondary *L*-amino acid residue with ninhydrin.

1.1) Reaction of primary *L*-amino acid residue and ninhydrin.



Scheme 78: Mechanism of primary *L*-amino acid residue with ninhydrin.

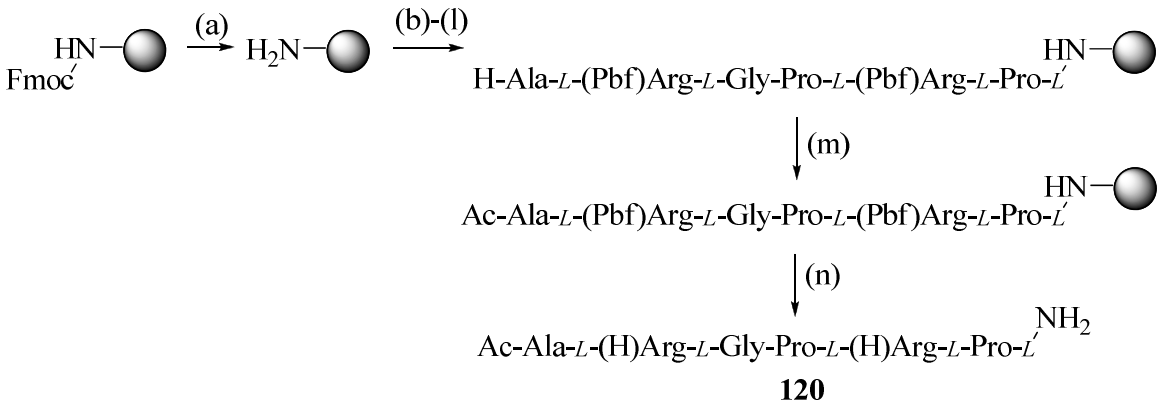
1.2) Reaction of secondary *L*-amino acid residue and ninhydrin.



Scheme 79: Mechanism of secondary *L*-amino acid residue with ninhydrin.

Appendix 2: Synthetic schemes of alanine scanning compounds **120**-**125** by solid phase peptide synthesis.

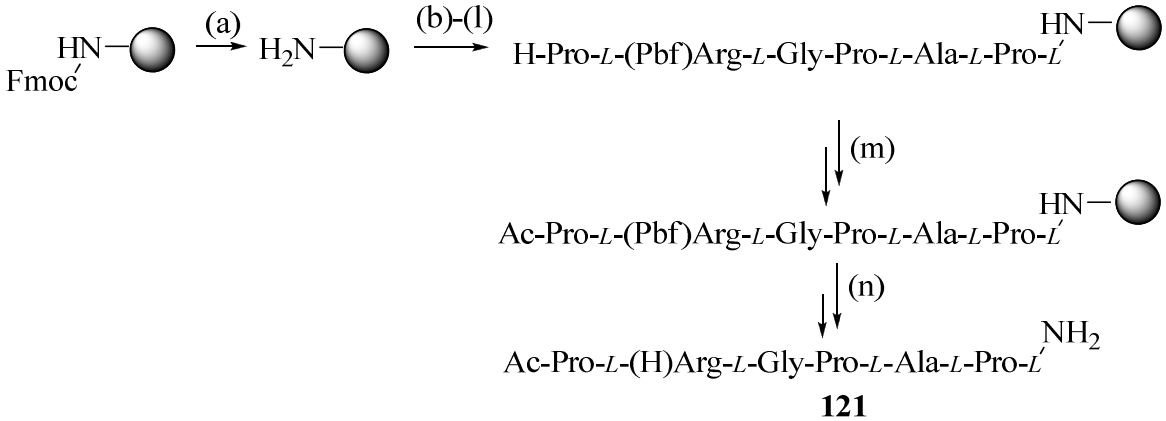
a) Synthesis of *N*-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH$_2$ 120.



120

a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-Gly-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Ala-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2).

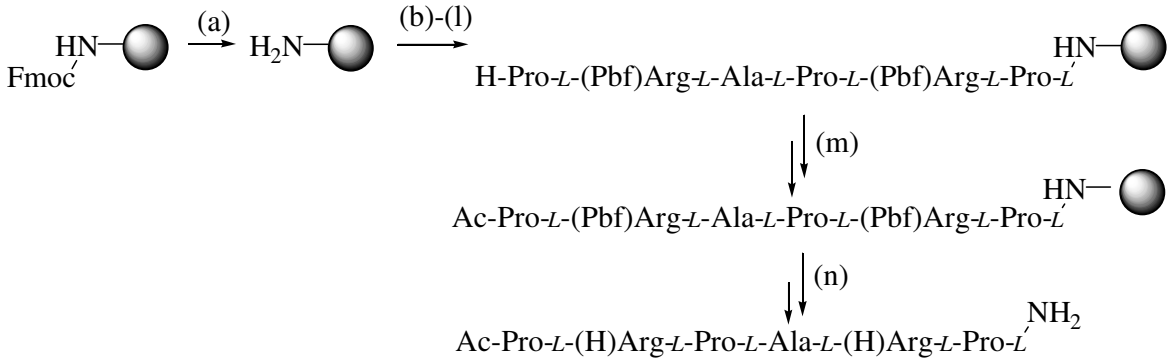
b) Synthesis of *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH$_2$ 121.



121

a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-Gly-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Ala-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2).

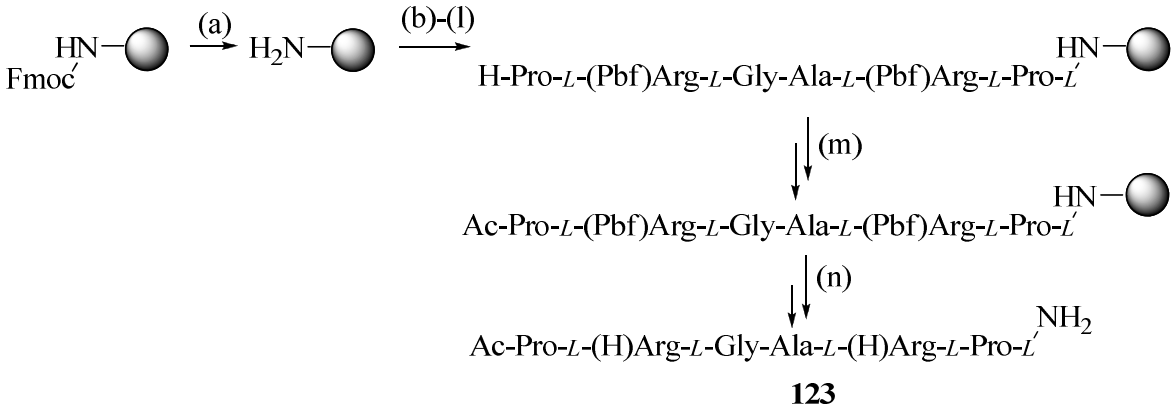
c) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 122.



122

a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-L-Ala-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2).

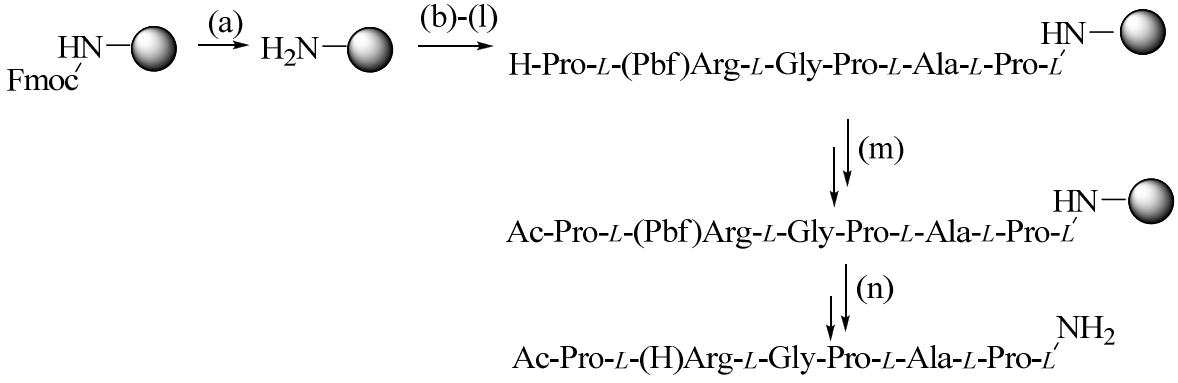
d) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2 123.



123

a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Ala-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-Gly-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2).

e) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2 124.

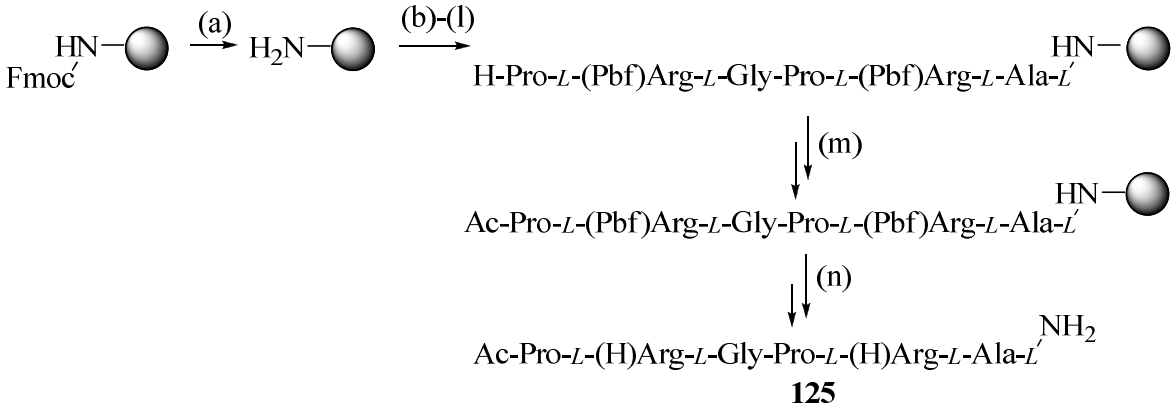


124

a) 20% piperidine/DMF, b) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Ala-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-Gly-

OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2).

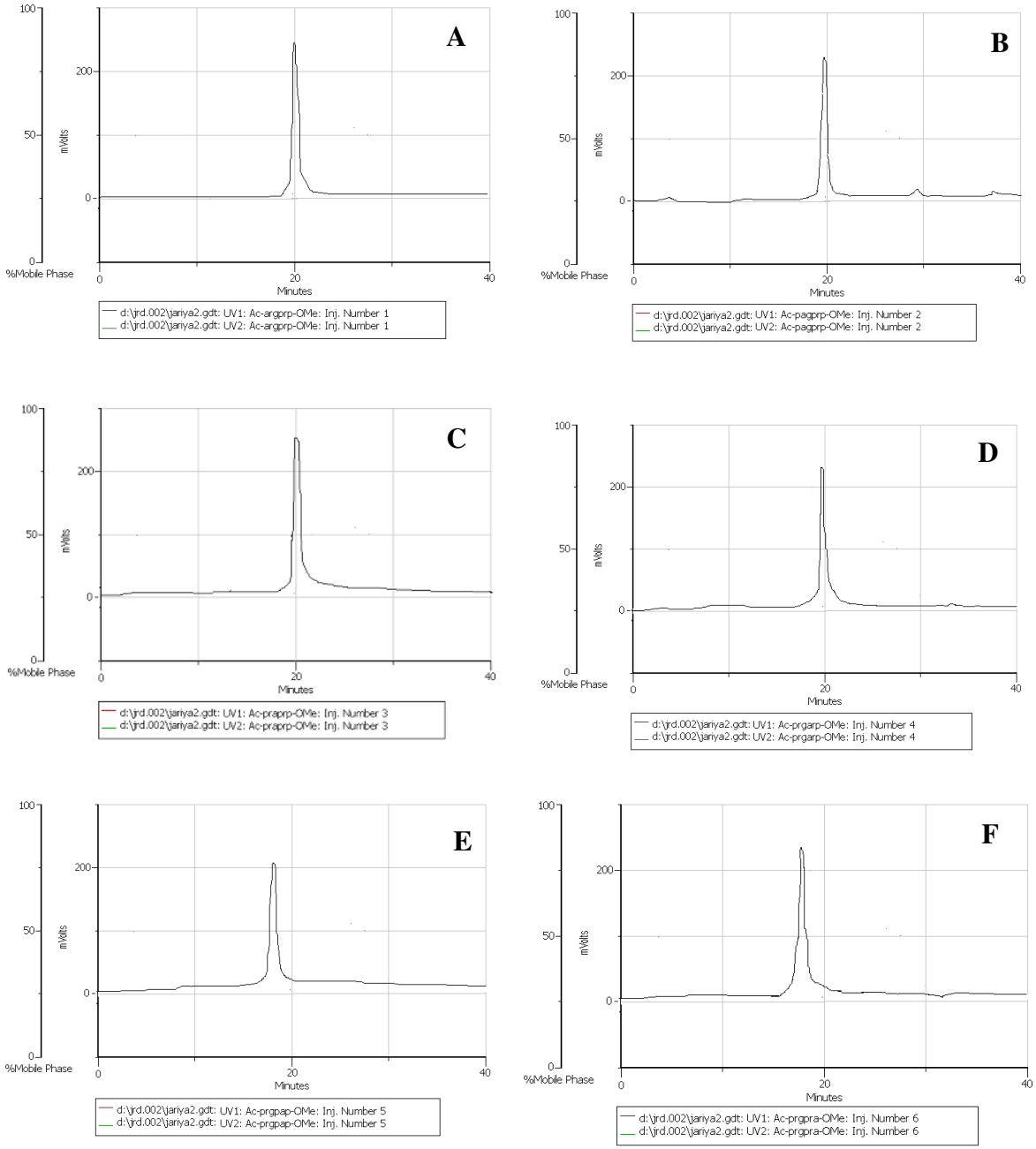
f) Synthesis of *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2 125.



125

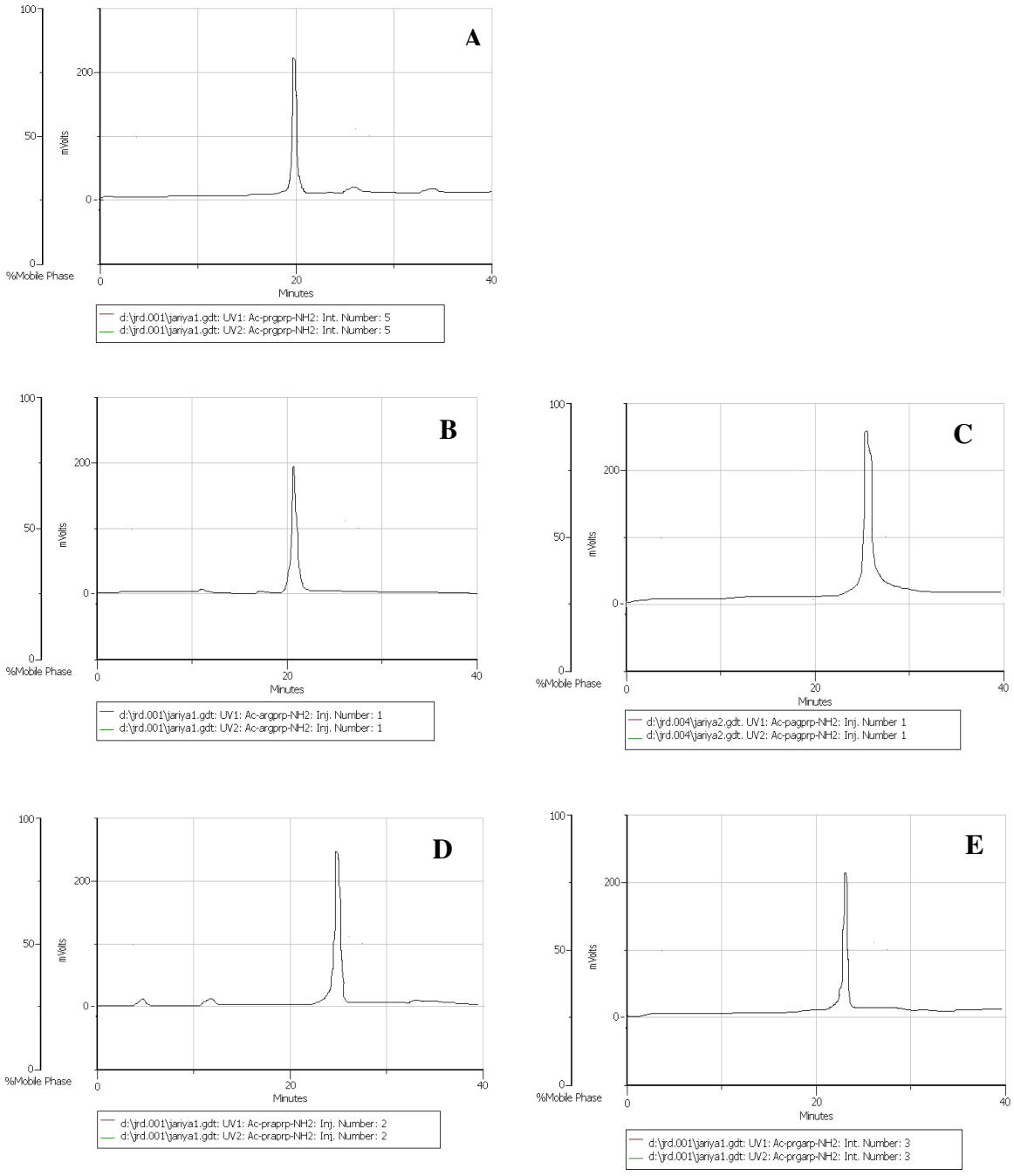
a) 20% piperidine/DMF, b) Fmoc-L-Ala-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, c) 20% piperidine/DMF, d) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, e) 20% piperidine/DMF, f) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, g) 20% piperidine/DMF, h) Fmoc-Gly-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, i) 20% piperidine/DMF, j) Fmoc-L-Arg(Pbf)-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, k) Fmoc-L-Pro-OH, DIC (3.0 equiv), HOBt (3.0 equiv), DIPEA (3.0 equiv), DMF, l) 20% piperidine/DMF, m) 50% Ac_2O/pyridine (3.0 equiv), n) TFA: TIS: H_2O (96:2:2).

Appendix 3: Single chromatogram of *L*-alanine scanning compounds which are synthesized by solution phase method.

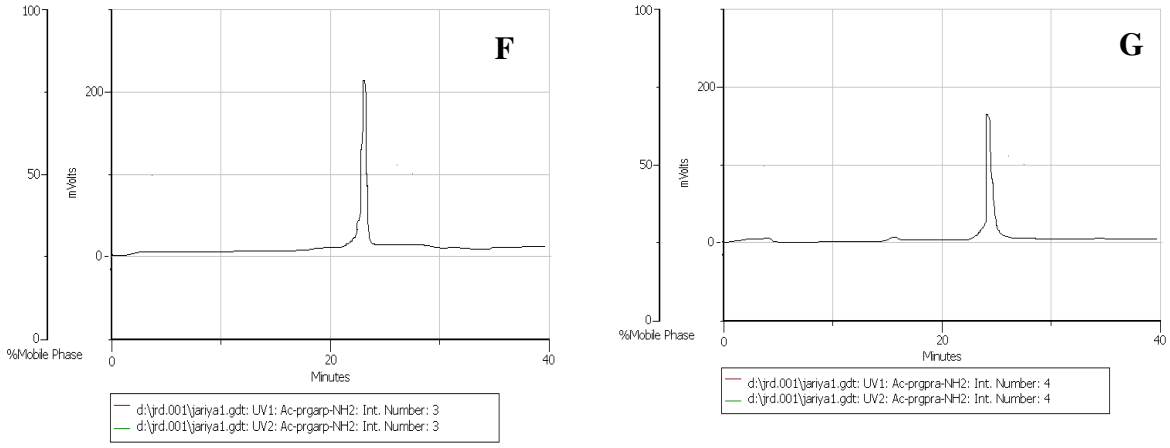


A) *N*-Ac-*L*-Ala-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **76**, B) *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **83**, C) *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro-OMe **90**, D) *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Ala-*L*-Arg(NO_2)-*L*-Pro-OMe **95**, E) *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-OMe **102**, F) *N*-Ac-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Ala-OMe **106.**

Appendix 4: Single chromatogram of parent hexapeptide **1** and its *L*-alanine scanning analogous which are synthesized by solid phase method.

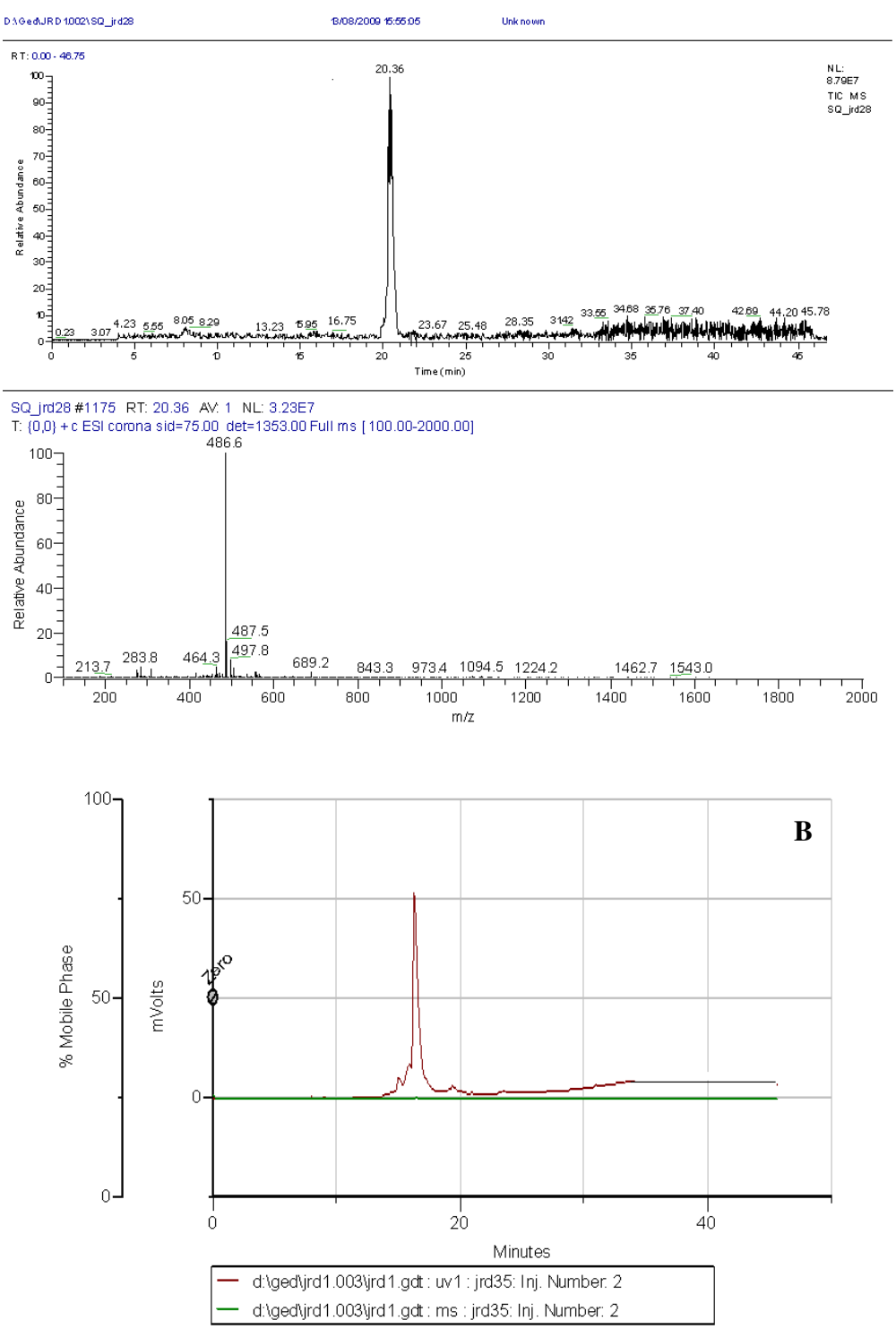


A) *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **1**, B) *N*-Ac-*L*-Ala-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **120**, C) *N*-Ac-*L*-Pro-*L*-Ala-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro- NH_2 **121**, D) *N*-Ac-*L*-Pro-*L*-Arg(H)-*L*-Ala-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2 **122**, E) *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Ala-*L*-Arg(H)-*L*-Pro-NH_2 **123**.



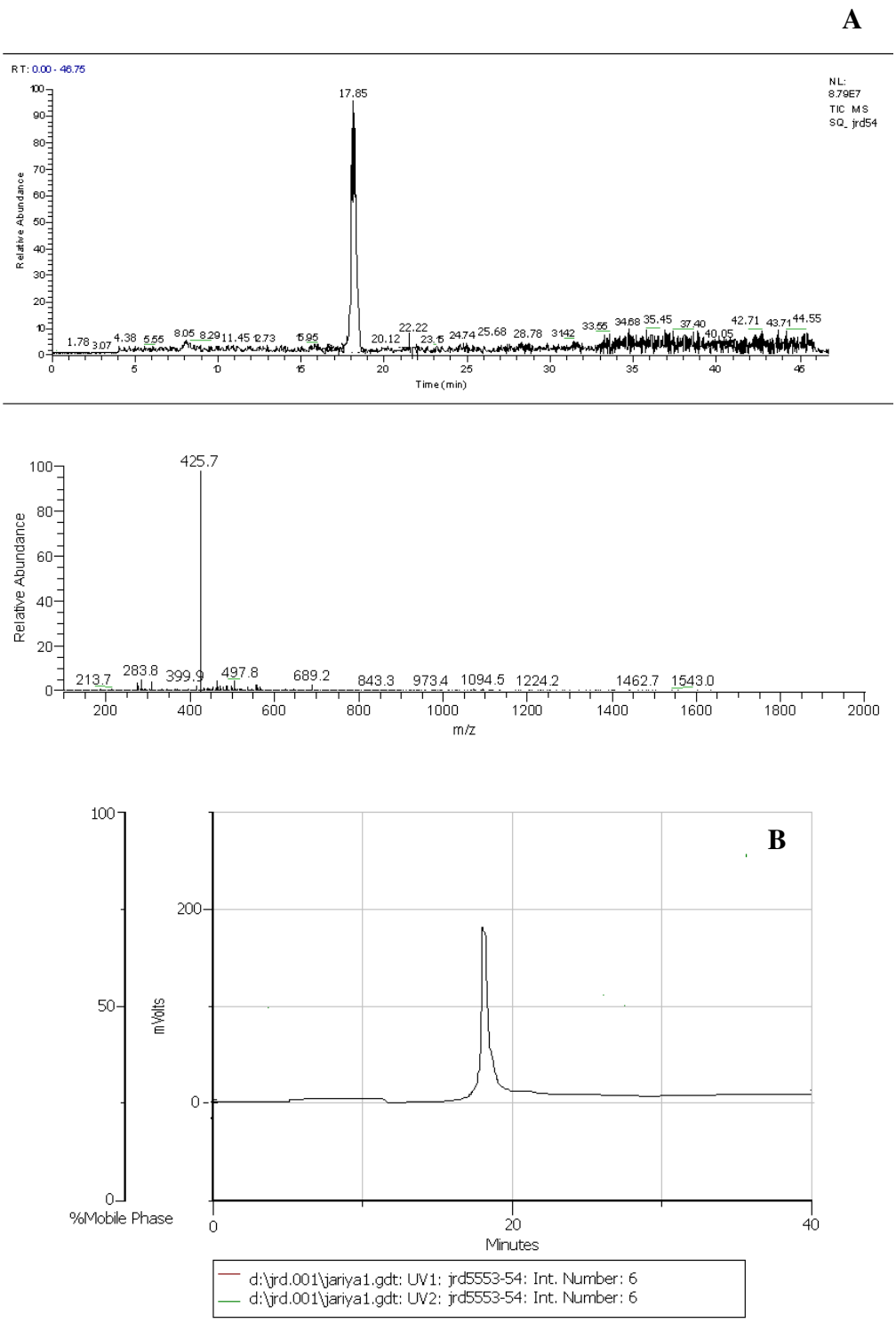
F) *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Ala-*L*-Pro-NH_2 **124**, G) *N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Ala-NH_2 **125.**

Appendix 5: HPLC chromatogram of cyclic peptide intermediate.



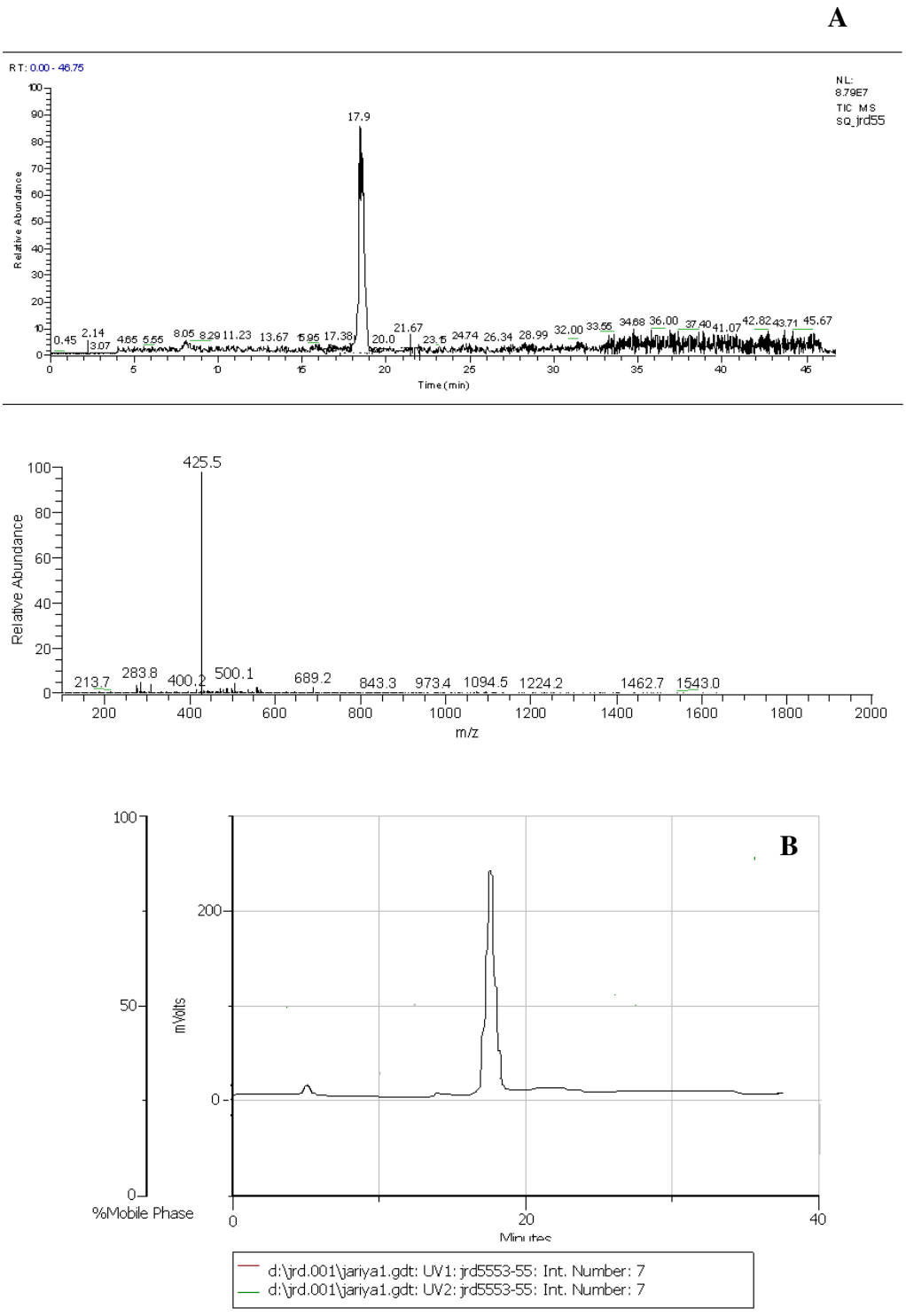
A) LCMS (ES^+) single chromatogram of cyclic compound, *N*-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-*L*-Arg(NO_2)-*L*-Pro-Gly-*L*-Pro-OMe, **163** at 486.6 $[(M\text{-}Boc)+2H)^{2+}]$, B) HPLC Single chromatogram of *N*-PEG-*N'*-Boc-*L*-Pro-*L*-Arg(NO_2)-Gly-*L*-Pro-*L*-Arg(NO_2)-*L*-Pro OMe **156**.

Appendix 6: HPLC chromatogram of cyclic peptide **161** (*N,C*-PEG-tethered-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro).



(A) HPLC Single chromatogram and (B) LCMS (ES^+) single chromatogram of cyclic compound **161** at 425.7 $[(M+2H)^{2+}]$.

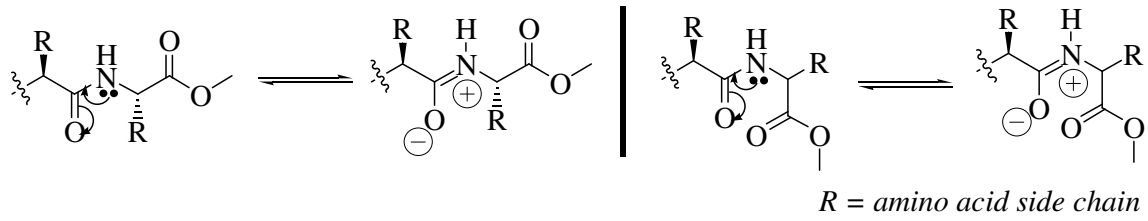
Appendix 7: HPLC chromatogram of cyclic peptide **168** (*N,C*-PEG-tethered-*L*-Pro-*L*-Arg(H)-*L*-Arg(H)-*L*-Pro-Gly-*L*-Pro).



(A) HPLC Single chromatogram and (B) LCMS (ES^+) single chromatogram of cyclic compound **168** at 425.5 $[(M+2H)^{2+}]$.

Appendix 8: Rotamers of peptides.

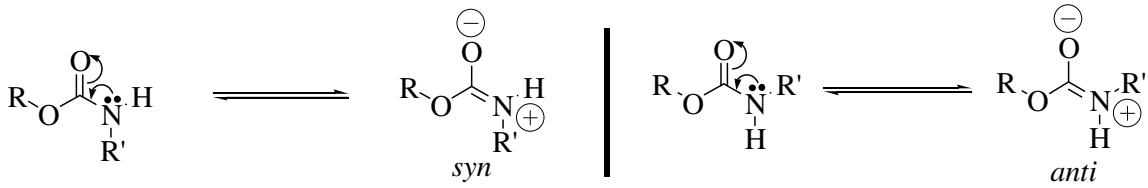
Rotamers of peptides can be occurred at amide bond as carbamate rotamers that can be observed in both of ^{1}H and ^{13}C NMR spectra (scheme 40 and 41).



R = amino acid side chain

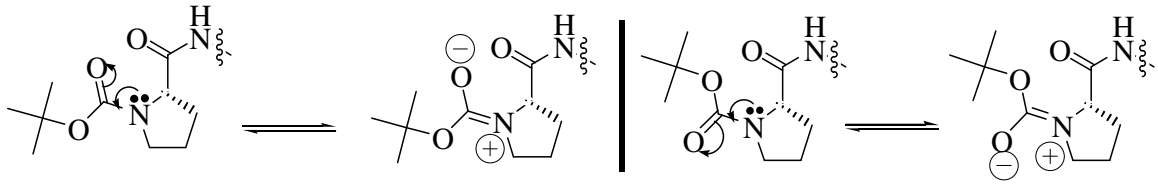
Scheme 80: Rotamer structures at amide bond in peptide chain.

Especially, *N*-Boc protecting group of peptide chain, carbamate rotamers can also be observed as *syn*- and *anti*-rotamers [77].



Scheme 81: Rotamers of carbamates.

Furthermore, *N*-Boc protecting group which is connected with *L*-proline residue, can be observed as the mixtures of rotamers in both of ^{1}H and ^{13}C NMR spectra (scheme 42).



Scheme 82: Rotamers of *N*-Boc protecting group of peptide chain.

Appendix 9: Clonogenic assay of ether contamination peptides against both of RT112 bladder cancer and fibroblast normal cells.

As results from solid phase peptide synthesis, there was ether contamination in the molecule of our linear peptides **1** and its analogue **120**-**125**. The ether was main reagent that used in precipitation step. This reagent could not be removed although we left our final products on high vacuum pump for overnight. The objective of this work was to observe effect of ether contamination with cells in biological assay. Then, the clonogenic assay was done under the same conditions that were developed against RT112 bladder cancer and MRC5-hTERT normal cells. Both of THR1 and parent peptide **1** (*N*-Ac-*L*-Pro-*L*-Arg(H)-Gly-*L*-Pro-*L*-Arg(H)-*L*-Pro-NH_2) were chosen to use in this assay and added some ether during the preparation of peptide solutions.

Clonogenic assay on day 29

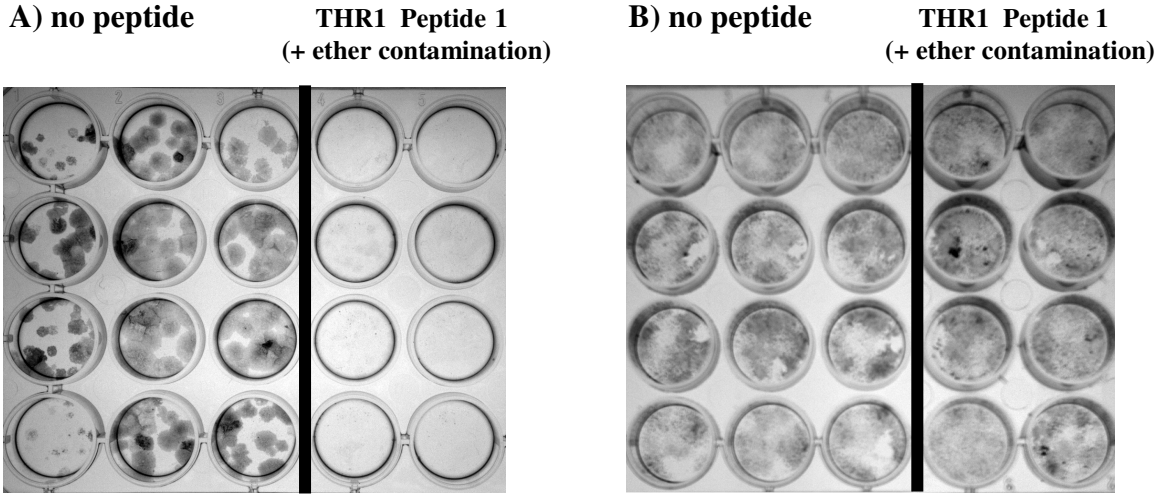


Figure 42A: *Pictures of colony forming of RT112 cells on day 29. The cells were trypsinized and seeded at 10 cells in each well and allowed to grow in Ham's F-12 medium for control experiment (500 µL),* ***Figure 42B:*** *Normal cells were trypsinized and seeded at 250 cells in each well and allowed to grow in DMEM for control experiment (500 µL). The effect of ether contamination was studied when the cells were exposed to peptide* ***1*** *(5 mM). The results were compared with THR1 (5 mM). Plates were kept at 37 ^{o}C in 5-10% CO_2 (depends on type of cells) in incubator before staining on day 29.*

As results on day 29 showed that synthetic peptide **1** could completely kill cancer cells on day 29 as same as THR1. Furthermore, both of these peptides did not have any effect with normal cells as well. We concluded that the ether contamination in our peptides during

precipitation step did not have any effect with our cells in biological assay. Because boiling point of ether (34.6 ºC) is lower than the temperature of incubator (37 ºC) then the trace of ether can be evaporated from system.

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